# Studies on Regulation of Gene Expression in Cultured Green Cells of Tobacco( Dissertation_全文 )

AUTHOR(S):

Takeda, Satomi

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SATOMI TAKEDA

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CONTENTS

## ABBREVIATIONS

| | |
|---|---|
| CAB | chlorophyll *a*/*b*-binding protein |
| CF1 | coupling factor 1 |
| Chl | chlorophyll |
| 2,4-D | 2,4-dichlorophenoxy-acetic acid |
| DCIP | 2,6-dichlorophenol indophenol |
| DCMU | 3-(3,4-dichloro-phenyl)-1,1-dimethyl-urea |
| 2D-PAGE | two dimensional polyacrylamide gel electrophoresis |
| EDTA | ethylenediaminetetraacetic acid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesufonic acid |
| LDS-PAGE | lithium dodecyl sulfate-polyacrylamide gel electrophoresis |
| LHC (I) | polypeptides of the light-harvesting chlorophyll-protein complex of PS I and LHC(II) of PS II |
| PA cells | photoautotrophically cultured cells |
| Pipes | Piperazine-N,N'-bis(2-ethanesulfonic acid) |
| PM cells | photomixotrophically cultured cells |
| PS I | photosystem I |
| PS II | photosystem II |
| PVDF | polyvinylidene difluoride |
| SDS | sodium dedecyl sulfate |
| Tricine | 2-hydroxymethyl-2-(N-glycine)-1,3-dihydroxy propane |
| Tris | tris(hydroxymethyl)aminomethane |

## INTRODUCTION

Photoautotrophism is one of the most distinctive attributes of plant cells carried out by chloroplasts. There are few studies on photosynthesis in plant cell culture systems, primarily because chlorophyll levels are usually quite low. However if the proper cells are selected and the culture system is properly manipulated, chloroplasts will develop along with the ability to perform photosynthesis. Photoautotrophic growth of in vitro cultured plant cells in a sugar-free medium but in the presence of $CO_2$-enriched air, was first achieved by Bergman in 1967 using tobacco suspension cultures (Bergman 1967). Since then, more than 25 different plant species have been established, with the exception of Gramineae.

Photoautotrophic (PA) plant cell suspension cultures simplify a complex plant organism to the cellular level with respect to its growth requirements and photosynthetic functions, including plastid differentiation. This facilitates the use of photoautotrophic cell culture to study the cellular basis of photosynthesis in higher plants. This paper summarizes the present understanding of photoautotrophic culture and describes the nature of my recent study.

*Establishment of photoautotrophic cell culture and development of chloroplasts*

Many PA cultures have been established by using a relatively lengthy procedure: Heterotrophic (H) callus cultures are initiated from a part of a plant in sugar-containing media. Green portions are then selectively transferred and photomixotrophic (PM) cell suspension cultures which contain chloroplasts are

initiated, The amount of sugar in the media is gradually reduced, until the cells are incubated in media which lack sugar. However, not all PA cultures have been initiated in this way, and many plant species could not be grown photoautotrophically. Most PA cultures require $CO_2$ levels of 1% or higher, as well as strong light intensity (8,000-12,000 lux, 100-300 $\mu E/m^2/s$) for growth. Their growth rates vary. Some cultures grow with a doubling time of two days (asparagus: Peel 1982) or four days (soy bean: Horn 1983, cotton: Blair et al. 1988), while other requires more than three weeks to double (perwincle: Tyler et al. 1986, *Hyoscyamus*: Yasuda et al. 1980).

These PA cells have well-developed chloroplasts with internal membranes organized in many stacks of grana, while plant cells which were cultured in sugar-containing media in the dark (H cells) contain amyloplasts which are heavily loaded with starch, and PM cells which were grown in the light contain chloroplasts with fewer grana.

The PA cells which contain chloroplasts with developed thylakoid systems generally have higher levels of chlorophyll than PM cells. However, most of the PA cells contain about 200 μgChl/gFW or less, which is about one-tenth the level found in leaves, although some cell lines (ex. SB-P, a soybean cell line: Horn 1983) have about 1500-2000 μgChl/gFW, which is equivalent to the chlorophyll content of leaves. In plant cell culture systems, however, there is no direct correlation between chlorophyll content and growth rate or photosynthetic capacity (Widholm 1992).



Photosynthetic capacities of PA cells

*Light reaction systems*

There are only a few reports on the light reaction systems in cultured cells (Sato et al. 1979, Dalton 1980). In these reports, the photosynthetic capacity of the light reaction systems, on a Chl basis, as measured by $O_2$ evolution, were about 30 to 550 μmole$O_2$/mgChl/h for PA cells, which is equivalent to the values for most mature $C_3$ plant leaves. However, on a fresh weight basis, most PA cells have much lower rates of photosynthetic $O_2$ evolution than leaves because of the lower Chl content of the PA cells.

*Dark respiration*

The rates of photosynthetic $O_2$ evolution are affected by those of dark respiratory $O_2$ uptake, which, as measured by $CO_2$ evolution, range from about 2 to 20 μmol$CO_2$/gFW/h or 9 to 90 μmol$CO_2$/mgChl/h for PA cells (Xu et al. 1988). Dark respiration rates of mature leaves, as measured by $CO_2$ evolution in the dark, were reported to be about 0.3-3 μmol$CO_2/m^2/s$, which can be converted to a Chl basis of about 3.6-36 μmol$CO_2$/mgChl/h, assuming a Chl content of about 300 $mg/m^2$, and to a fresh weight basis of 10.8-108 μmol$CO_2$/gFW/h, assuming a value of 100 $gFW/m^2$. Thus, the dark respiration rates are of the same order on a Chl basis, while the rates of the cultured cells are lower if calculated on a FW basis, although the difference was small compared to that between the respective amounts of Chl on a FW basis.

*Carboxylation reactions*

The light fixation rates of $^{14}CO_2$ in PA cells, calculated as μmol $CO_2$ fixed per mg Chl per hour, range from 9-28 for *Amaranthus*

*powellii* (Xu et al. 1988) to 132 for *Nicotiana tabacum* (Nishida et al. 1980). The dark fixation rates of $CO_2$ on a Chl basis range from 1.3 for *Amaranthus cruentus* to 10 for *Chenopodium rubrum*, which were 3-30% of the light $CO_2$ fixation rates of the respective PA cells (Xu et al. 1988).

One of the remarkable characteristics of carbon fixation in PA and PM cells is that high $^{14}C$ labeling can be observed in $C_4$-fixation products, such as aspartate and, in particular, malate, aside from Calvin cycle derivatives (Hüsemann et al. 1979, Nishida et al. 1980, Sato et al. 1980, Seeni and Gnanam 1982). Moreover, in many PA cell culture systems, phospho*enol*pyruvate carboxylase (PEPCase) activities were higher than ribulose 1,5-bisphosphate carboxylase (RuBPCase) activities. In addition, the ratio of RuBPCase and PEPCase activities changes during the growth cycle; PEPCase activities, as well as the concentration of PEPCase, are highest during the exponential growth phase, while the activities and concentration of RuBPCase decrease during that phase. In contrast, PEPCase activities decrease and RuBPCase activities increase during the stationary phase (Nato et al. 1985).

Since most PA cells have higher levels of PEPCase, on a Chl basis, than leaves and the activities are higher during the exponential phase when the respiration rate is high and cells are actively dividing and synthesizing structural components, PEPCase is believed to feed carbon to the tricarboxylic acid cycle for the production of amino acid, and may be involved in the other biosyntheses which would be needed during this phase (anaplerotic $CO_2$ fixation) (Nishida et al. 1980, Sato et al. 1980, Roger 1987, Sato et al. 1988).



On the other hand, the activities of RuBPCase in PA cultured cells are much lower than those in leaves. Goldstein and Widholm (1990) reported that *in vivo* activation levels of RuBPCase in PA cultured cells were also lower than those in leaves. They found that if RuBPCase was extracted by conventional methods, i.e., cells are taken directly from flasks under a relatively low light intensity (approximately 250 $\mu E/m^2/s$), the activities prior to activation with high $Mg^{++}$ and $HCO_3^-$ (initial activities) were only 16-56% of the activities after activation (total activities), while the initial activities of RuBPCase in leaf extracts were usually more than 90% of the total activities. However, Roeske et al. (1989) showed that the RuBPCase in PA cultures could be activated to higher levels (93 to 94%) with a 5-min illumination prior to extracting RuBPCase.

Many Studies have demonstrated that PA cultures perform photorespiration by showing that photosynthesis in PA cultures is inhibited by increased $O_2$ levels, that this inhibition can be reversed by increasing $CO_2$, and that $^{14}CO_2$ is incorporated into glycine and serine (Sato et al. 1979, Roeske et al. 1989, Rey et al. 1990).

*$CO_2$ compensation points*

Most photosynthetic cultures require $CO_2$-enriched air (1-2%, v/v) for growth. This might be explained by a low carbonic anhydrase activity, which, by catalyzing the dehydration of bicarbonate, could reduce the availability of $CO_2$ as a fixation substrate (Tsuzuki et al. 1981). However, a cotton cell line which can grow under ambient $CO_2$ levels was recently established. Furthermore, this cell line does not contain carbonic anhydrase, as measured by enzyme assay and immunological methods, while

other PA cells which require $CO_2$-enriched air do contain carbonic anhydrase at levels comparable to that in spinach leaves (Roeske et al. 1989). Therefore, the carbonic anhydrase activity does not appear to be related to the need for $CO_2$. While RuBPCase activity, high mitochondrial respiration and photorespiration are possible explanations, the precise reason why most PA cultures require elevated $CO_2$ levels for growth is presently unclear.

As described above, PA cells show several characteristics which differ from leaves. These differences might reflect the specific gene expression of cultured cells. Direct comparison of the characteristics of PA cells and green leaves (mesophyll cells) which posses the same metabolic functions, i.e. photosynthesis, should provide information to help elucidate the mechanism which controls gene expression in cultured cells. In this study, I first investigated the structure of chloroplasts and their photosynthetic activities in cultured green cells as compared to those in leaf cells (Chapter I). I also investigated the effects of several herbicides with various modes of action on plant metabolism on cultured tobacco cells and seedlings (Chapter II). These investigations indicated that photoautotrophic cultured tobacco cells have functions similar to those of mesophyll cells, although these cells are relatively large and their chlorophyll content, on a fresh weight basis, and photosynthetic activities are low. The investigation of protein accumulation in photoautotrophic cultured tobacco cells clearly showed that cultured cells are highly stressed (Chapter III). Furthermore, the isolation of cDNA of a stress-induced protein with some sequence homology with osmotin, a salt-inducible protein, and the



characterization of the regulation of its gene expression indicated that ethylene, a stress hormone in plants is a potent hormone which regulates gene expression in cultured cells (Chapter IV).

CHAPTER I

# PHOTOSYNTHETIC CHARACTERISTICS OF CULTURED GREEN CELLS OF TOBACCO

As described in the Introduction, cultured green cells have been shown to posses several characteristics which differ from those of mesophyll cells. However, these earlier observations are mainly limited to PM cells which grow in the presence of organic carbon sources. This chapter discusses the ultrastructure and photochemical activities of truly photoautotrophic cells.

Many studies using photomixotrophically cultured green cells have shown that photosynthetic activities increase with the development of plastid internal membrane systems during the greening process. Gillott et al. (1991) reported that amyloplasts in heterotrophically cultured cells differentiated into chloroplasts under continuous light, and that chloroplasts in photomixotrophically cultured cells dedifferentiated to amyloplasts as the organized thylakoid network was lost and starch accumulated in the dark. Similar ultrastructural changes of plastids have been observed in PM tobacco cells during greening (Brangeon and Nato 1981). Plastid ultrastructural changes have also been observed during cell growth (*Nicotiana tabacum* L. cv. Xanthi: Nato et al. 1977, spinach: Aguettaz et al. 1987). However, all of these studies on ultrastructural changes in plastids involve qualitative analyses; no quantitative analysis has been performed for a comparison with leaf cells.

Several studies on the photosynthetic capacities of photomixotrophic cultured green cells have been reported, but



information regarding the characteristics of their photochemical activities was very limited (Sato et al. 1979, Seeni and Gnanam 1982). Therefore, I characterized the changes in chloroplast ultrastructure, chlorophyll content, chloroplast number, photochemical reactions, and thylakoid membrane polypeptide composition in PA cells in comparison with those of PM cells and leaf cells of tobacco.

## MATERIALS AND METHODS

### *Plant and cell materials*

Photoautotrophic cultures of tobacco (*Nicotiana tabacum* cv. Samsun NN) were maintained in modified Linsmaier and Skoog liquid media without sucrose in air enriched with $CO_2$ (1-2%) as described previously (Yamada and Sato 1978), except that two-tier flasks were used as described Hüsemann and Barz (1977). Photomixotrophically cultured tobacco cells were grown in the same media containing 3% sucrose (w/v). PA and PM cells were subcultured every three weeks and every two weeks, respectively. Both cells were cultured at 25±2 °C with reciprocal shaking (100 rpm) under continuous light (100-150 $\mu E/m^2/s$). Intact tobacco plants were watered daily in soil in a greenhouse under natural illumination. The plants were used two to three months after germination when they had reached a height of 30 cm.

### *Growth measurement*

Cultured cells were collected by vacuum filtration, washed three times with distilled water, and weighed to determine fresh weight.

*Measurements of chlorophyll contents*

Chl was extracted from the leaves and cells with aqueous acetone (80%, v/v). Total Chl, Chl*a* and Chl*b* were determined spectrophotometrically (Yamada and Sato 1978).

*Electron microscopy*

Cells or leaf segments were suspended in 2% (v/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7.0), and fixed for one hour at room temperature. Fixed materials were washed with 0.05 M phosphate buffer (pH 7.0) three times and post-fixed in 2% (w/v) $OsO_4$ in 0.05 M phosphate buffer (pH 7.0) at 4 °C overnight. The post-fixed samples were dehydrated through a ascending series of propylene oxide and infiltrated in Spurr's embedding media. Samples were finally embedded in flat molds, and polymerized at 70 °C. Thin sections (60-90 nm) were stained with uranyl acetate and lead citrate, and examined with a Hitachi H400H transmission electron microscope. For light microscopy, 0.2-0.25 μm sections were stained with toluidine blue.

*Measurements of numbers of cells and chloroplasts*

The cell numbers in certain weights of cells were determined by the method of Fosket (1968) with slight modifications. Certain weights of leaf section or cultured cells were macerated in 5% chromic acid solution. The number of chloroplast per cell was counted under a microscope after the tissue was squashed (Tsuji et al. 1979).



*Isolation of chloroplasts*

To measure photochemical reaction activity, chloroplasts were isolated as described elsewhere (Sato et al. 1979) with slight modifications. All procedures were carried out at 0-4 °C. Chilled materials (ca. 30 g for cultured cells, ca. 5 g for green leaves) were homogenized in 60 ml of Tricine-sucrose buffer (20 mM Tricine-NaOH (pH 7.8), 0.4 M sucrose, 10 mM NaCl, 2 mM $MgCl_2$, 0.1% BSA) together with a small amount of polyvinylpyrrolidone in a Waring blender for either 30 s (cultured cells) or 10 s (leaves). After the homogenates were filtered through eight layers of gauze, the filtrates were centrifuged at 400 x g for 2 min, and the chloroplasts were sedimented from the supernatants at 2,000 x g for 8 min. One ml of 2 x Tricine-sucrose buffer was added to the pelleted chloroplasts suspended in 1 ml of distilled water for 1 min, and the suspensions were stored on ice until use.

*Assay of activities of photosystem I (PS I) and of the Hill reaction in isolated chloroplasts*

The methyl viologen-mediated oxygen uptake in PS I activity (Epel and Neumann 1973) was measured with DCIP/ascorbate as the electron donor in the presence of DCMU. The reaction mixture (1 ml) contained 50 mM Tris-HCl (pH 7.2), 0.5 mM sodium ascorbate, 0.1 mM methyl viologen, 50 μM DCIP, 1 mM $NaN_3$, 10 μM DCMU and the chloroplast fragments (10 μg Chl). The Hill reaction activity was measured by the evolution of oxygen in the uncoupled condition with ferricyanide as the electron acceptor. This reaction mixture (1 ml) contained 50 mM sodium phosphate (pH 7.8), 10 mM NaCl, 1 mM potassium ferricyanide, 1 mM $NH_4Cl$, and the chloroplast fragments (10 μg Chl). Hill reaction

activity coupled with photophosphorylation was measured under the conditions described above except that the $NH_4Cl$ was omitted. All measurements were made with a water-jacketed oxygen electrode (Hansateck, U. K. ) at 25 ℃ under 100,000 lux.

*Analyses of thylakoid membrane polypeptides*

To analyze the thylakoid membrane polypeptides, the chloroplasts were purified as follows. Cooled leaves (ca. 30 g), or cultured cells (ca. 100 g), were homogenized in a Waring blender for 2 s in 200 ml of 50 mM Mes-NaOH buffer (pH6.1), containing 0.33 M sorbitol, 2 mM $Na_2$-EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 20 mM NaCl, 2 mM isoascorbic acid, 0.1% BSA and 1% polyvinylpyrrolidone, with slight modifications to the method described by Jensen and Bassham (1966). The resulting homogenates were quickly filtered through four layers of gauze and one layer of Miracloth (Behring Diagnostics, La Jolla, CA). Chloroplasts were collected by centrifugation at 2,500 x g for 90 s. These 'crude' chloroplast preparations were re-suspended in a small amount of HEPES-sorbitol buffer (50 mM HEPES-NaOH (pH6.8), 0.33 M sorbitol, 2 mM $Na_2$-EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $Na_2$-pyrophosphate, 1 mM phenylmethylsulfonyl-fluoride, 5 mM ε-aminocaproic acid and 1 mM benzamidine-HCl), then purified by Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation. Two or three milliliters of a preparation were layered on a 40 ml Percoll linear gradient solution (previously formed by self-generating centrifugation) containing HEPES-sorbitol buffer. The chloroplast fractions in the gradient were sedimented by centrifugation at 2,000 x g for 10



min, after dilution of the chloroplast fraction with HEPES-sorbitol buffer.

Thylakoid membrane polypeptides were analyzed by LDS-PAGE, essentially according to the method of Delepelaire and Chua (1979). The chloroplast preparation (10 μg) was mixed with an LDS-loading buffer (Delepelaire and Chua 1979) and applied to the 12% polyacrylamide gel. For detailed identification of the membrane polypeptides, the thylakoids were fractionated by chloroform/methanol extraction according to Chua et al. (1975). Chloroplasts were heated in the loading buffer to identify the presence of thylakoid polypeptides.

## RESULTS

*Changes in fresh weight, Chl contents, cell numbers, cell volume and chloroplast number during the growth of cultured cells and the development of leaves*

Changes in cell number, fresh weight and Chl content of PA cells, PM cells, and leaves are shown in Fig. 1. The fresh weight of the PA cells increased gradually after a lag of one week. PM cells grew more rapidly and showed normal sigmoidal growth kinetics. The fresh weight of the leaves increased during leaf development and decreased with senescence. The Chl content of leaves, on a per cell or fresh weight basis, peaked earlier than the fresh weight. In contrast, the Chl contents of the cultured cells were relatively constant, except for a drop during the lag phase, while the Chl content on a fresh weight basis was about 10-fold less than that for cells of expanding leaves (leaf positions 9 to 15).

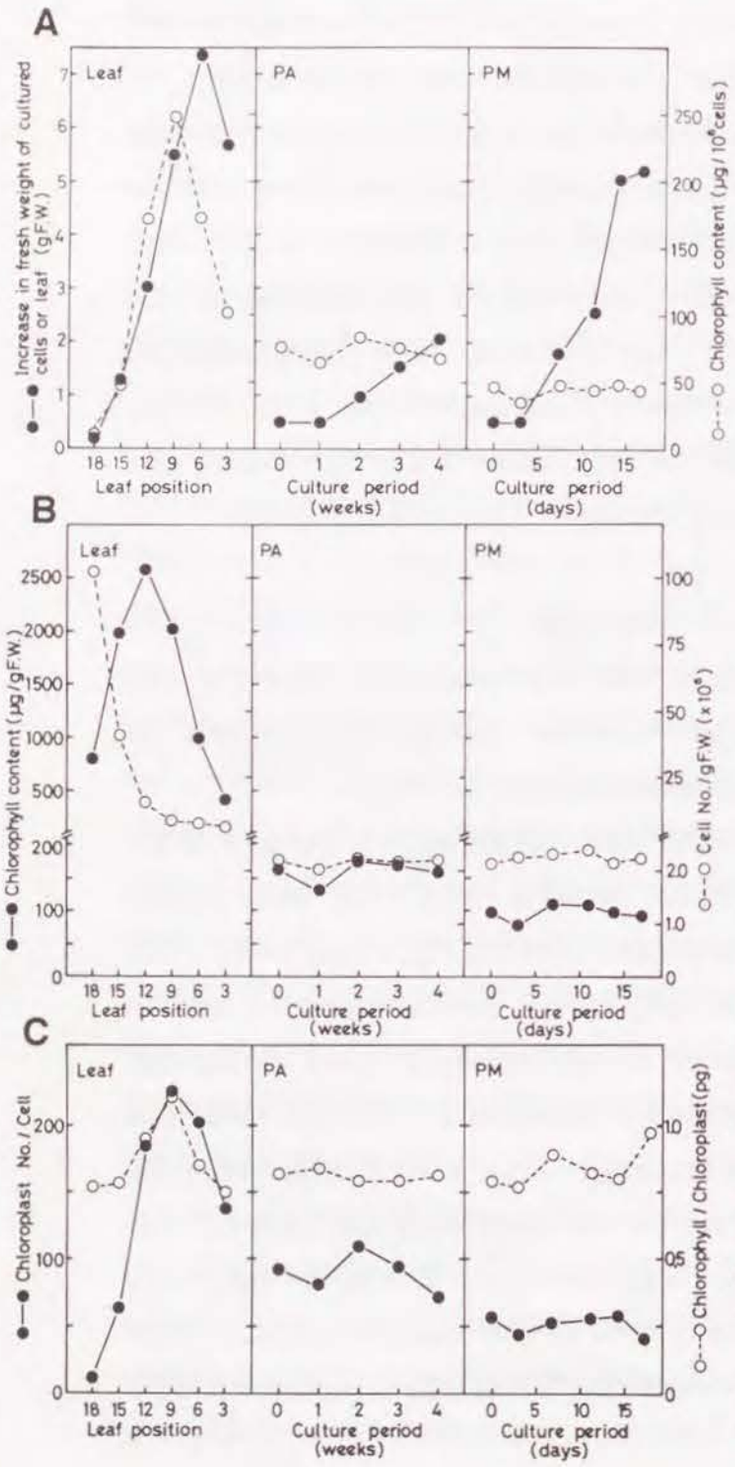



Fig. 1. Characteristics of chloroplast development in cultured green cells and leaves of tobacco. A. Increases in fresh weights and changes in Chl contents per cell of leaves and suspension cultured cells of tobacco. Leaf position is determined by counting from the primary leaf. B. Changes in Chl content and cell number, on a fresh weight basis, during the growth of leaves and cultured cells of tobacco. C. Changes in chloroplast number per cell and Chl content per chloroplast during the growth of leaves and cultured cells of tobacco.



The cell number per gram fresh weight of expanding leaves (leaf positions 9 to 15) was about 4- to 20- fold that in cultured cells (Fig.1B) which indicates that the cell volume of a cultured tobacco cell is much larger than that of a tobacco mesophyll cell. This difference in cell volume by cell type was confirmed by microscopy (data not shown). The ratio of cell number per unit weight of cells (tissues) also indicates that the volumes of the cultured cells were nearly constant throughout the growth cycle, whereas the volume of a mesophyll cell was small in young leaves but increased with development (Fig.1B).

The change in the number of chloroplasts per cell was similar to the change in the Chl content per cell in both the cultured and mesophyll cells (Figs.1A and C). This indicates that the Chl content per chloroplast was nearly constant during growth for the cultured and mesophyll cells, except that the Chl content per chloroplast increased after 14 days in PM cells and that in mesophyll cells showed a small increase just before full development of the leaf (Fig. 1C).

These results indicate that the larger cell volume of cultured cells is the main factor in the difference between the chlorophyll contents of mesophyll and cultured cells. The chloroplast number per mesophyll cell, however, determined the Chl content of the tissue during growth.

*Ultrastructure of nuclei and chloroplasts*

Nuclei: The nuclei in cultured cells were much larger (ca.10-20μm in diameter) than those of mesophyll cells(ca.5-7μm in diameter) (Figs. 2A, 3B and 4A). Polyploidy might explain the enlargement of nuclei in cultured cells, while other factors, such

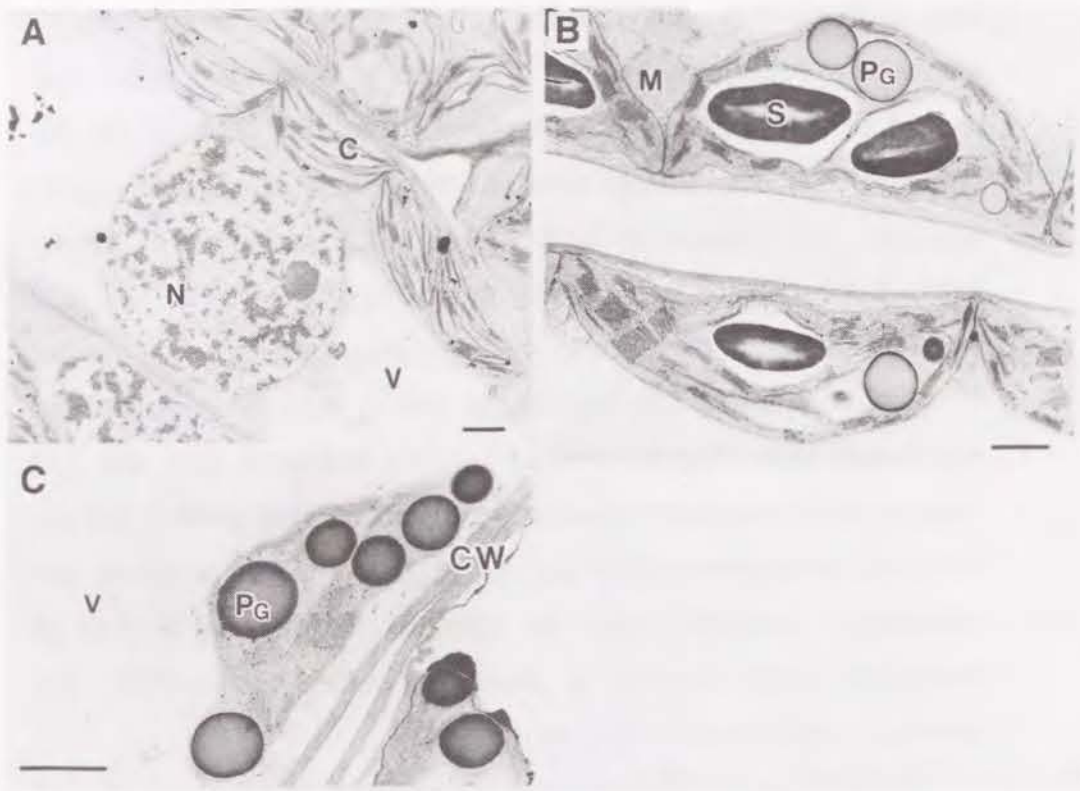



Fig. 2. Ultrastructure of tobacco mesophyll cells.
A. Young leaf (leaf position 12). B. Mature leaf (leaf position 9). C. Old leaf (leaf position 6). Abbreviations: N=nucleus, C=chloroplast, PG=plastoglobule, V=vacuole, M=mitochondrion, S=starch, CW=cell wall. Bar=1μm.



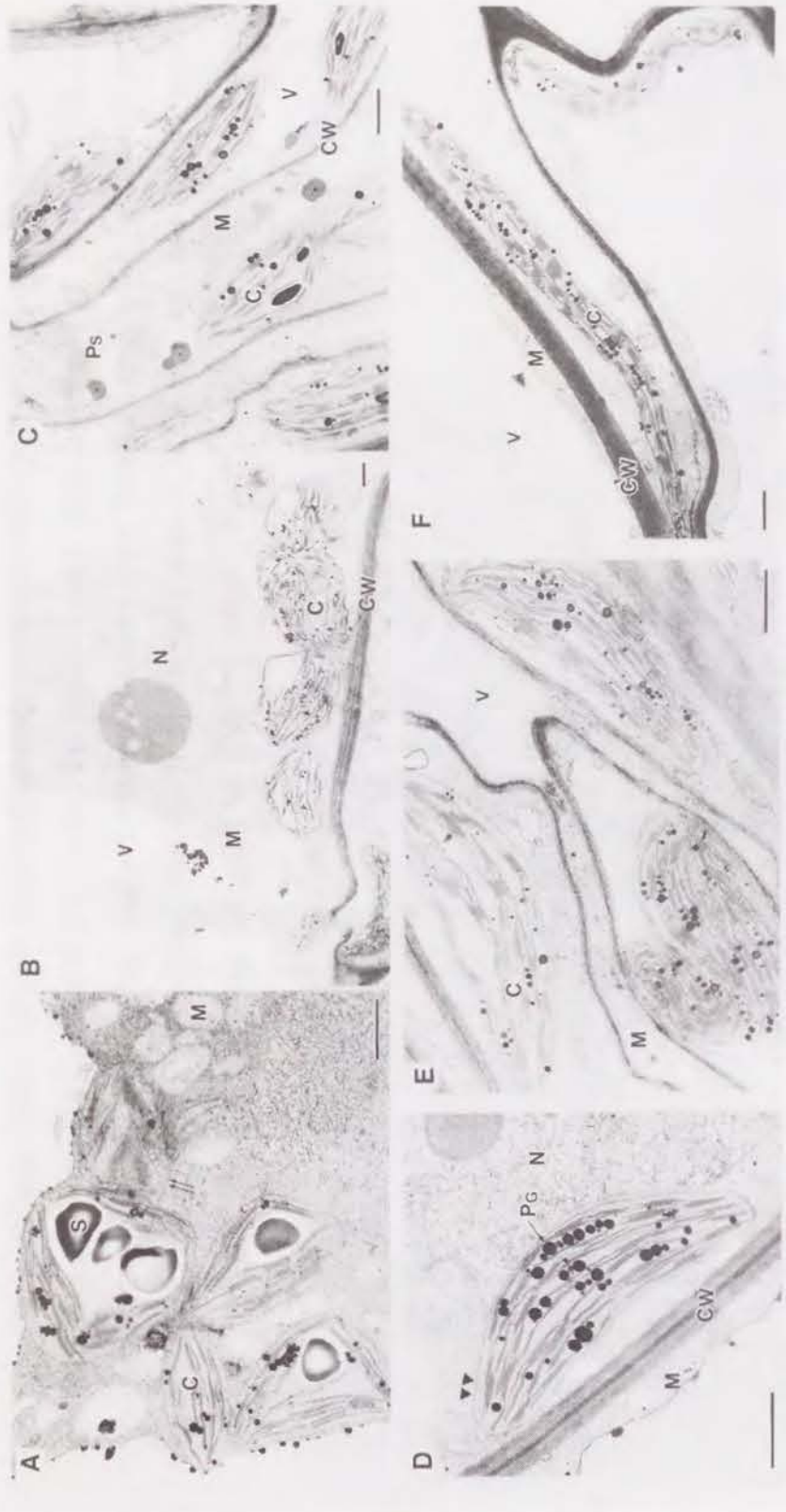



Fig. 3. Ultrastructural change of PA cells of tobacco during growth cycle. A. 3 days of growth; several types of plastids were present: chloroplasts and amoeboid-shaped plastids (double arrow). B. 7 days of growth; a chloroplast was dividing into four. C. 14 days of growth; chloroplasts began to elongate. D. 21 days of growth; invaginations of envelope (▲) were present in the chloroplast. E. 28 days of culture. F. 35 days of growth; many elongated chloroplasts are observed. Ps=peroxysome. Other abbreviations are same as Fig. 2. Bar=1μm.

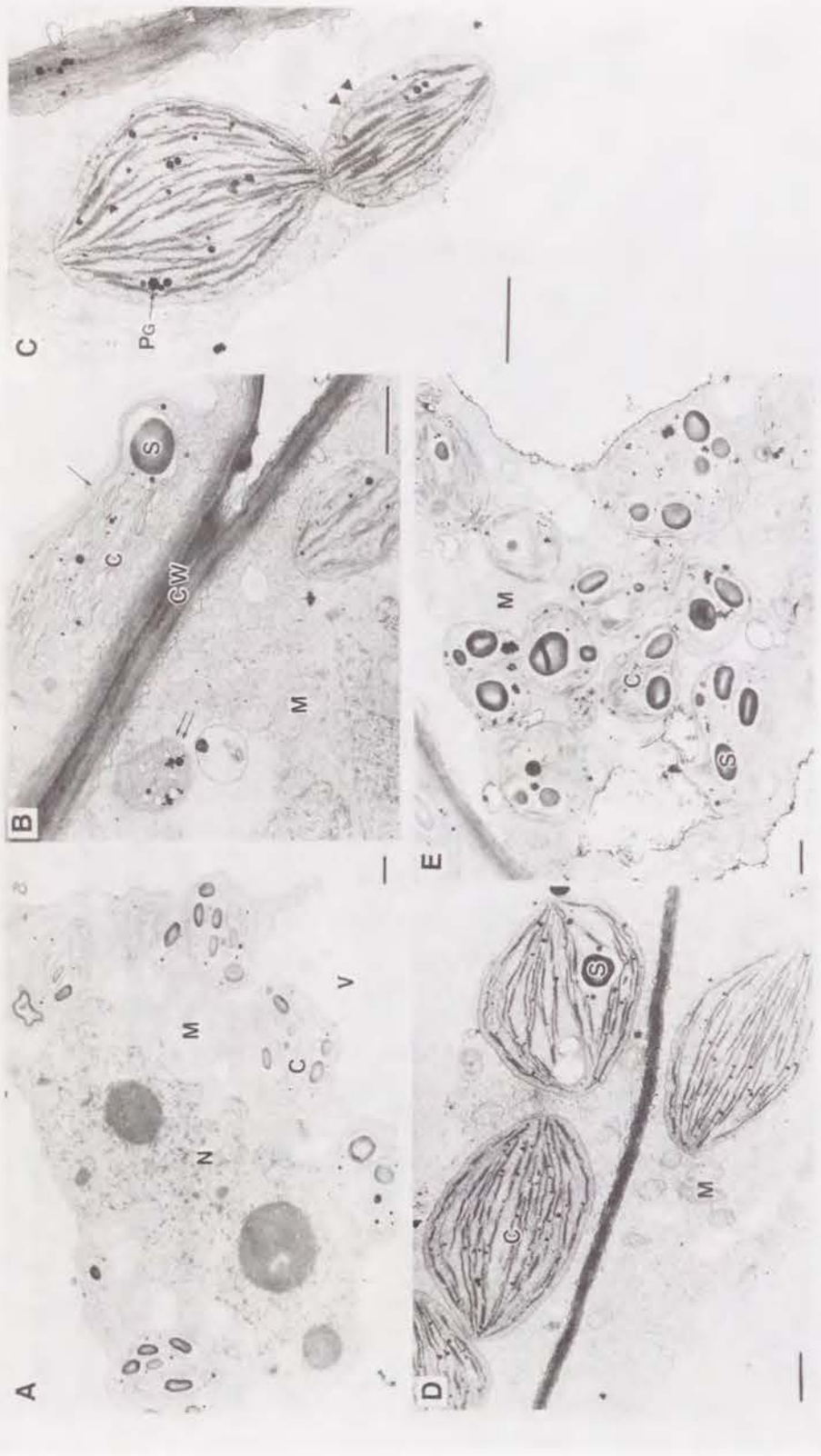



Fig. 4. Ultrastructural changes of PM cells of tobacco during growth cycle. A. 1 day of growth; chloroplasts were preferentially located near the nucleus. B. 3 days of growth; amoeboid-shaped plastid (double arrow) and chloroplast with swollen lamellae (arrow) were present. C. 7 days of growth; many dividing chloroplasts were present at this stage. D. 10 days of growth E. 14 days of growth; many plastids with fragmented cisternae and numerous vesicles were obser Abbreviations are the same as Fig. 2. Bar=1μm.



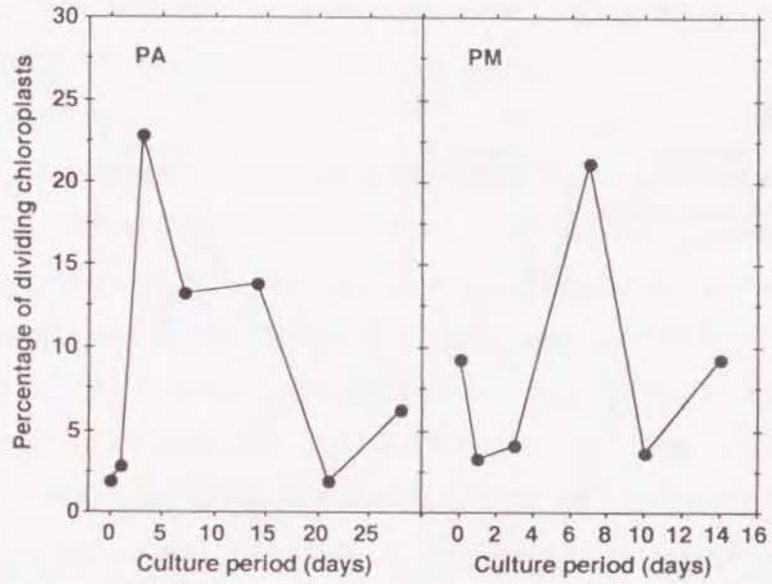



Fig. 5. Changes in the percentage of dividing chloroplasts. Percentages were counted on electron micrographs of sections of 50 to 70 chloroplasts in cultured tobacco cells.

as auxin or large cell size may also play a role. The shapes of nuclei in cultured cells were usually more irregular and nuclei themselves were more surrounded by chloroplasts than those of mesophyll cells.

Chloroplasts: Chloroplasts in PA cells were similar in shape to those in mesophyll cells, although those in PA cells elongated during the late growth stage (Figs. 3E and F). On the other hand, chloroplasts in PM cells were more globular than those in other cells (Fig. 4). Interestingly, while dividing chloroplasts were often observed in cultured cells (Figs. 3B and 4C), they were rarely observed in green leaves. The chloroplasts in PA cells began to

divide 3 days after inoculation, while in PM cells they began to divide 7 days after inoculation (Fig. 5). Chloroplasts usually

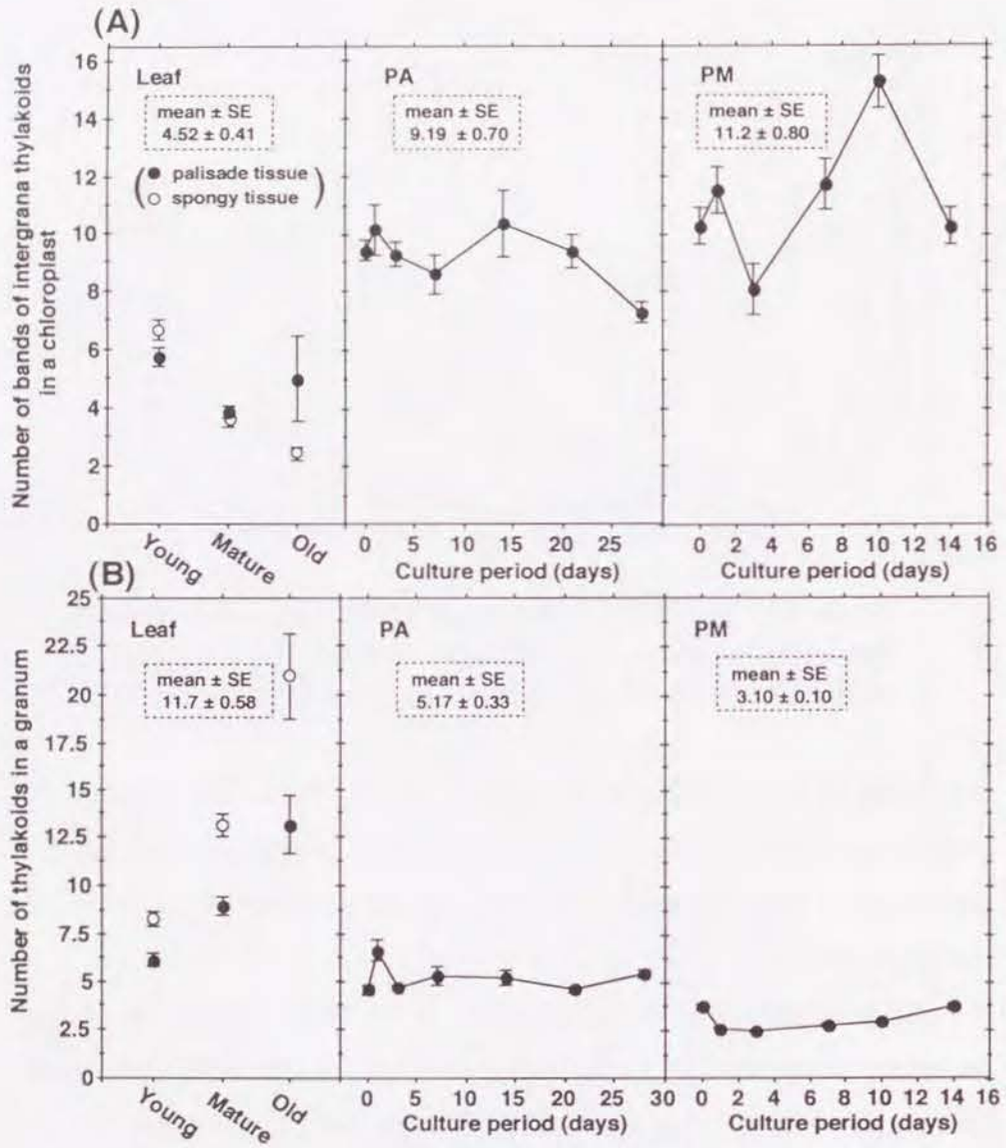



Fig. 6. Quantitative changes in the thylakoid membrane system in a chloroplast during the growth of leaves and cultured cells of tobacco. The number of bands of intergrana thylakoids in a chloroplast (A) and the number of thylakoids in a granum (B) were counted on electron micrographs of sections of 15 chloroplasts in cells. Vertical bars indicate the standard error.



divided into two daughter chloroplasts, as in the division of prokaryotic cells (Fig. 4C). However, in some cases and especially in PA cells, elongated chloroplasts were fragmented into many daughter chloroplasts by constriction (Fig. 3B).

Inner membrane system of chloroplasts: The development of inner chloroplast membrane systems was measured in terms of the number of bands of intergrana thylakoids in a chloroplast ('band' refers to a set of thylakoids interconnected by stacking), and by the number of thylakoids in a granum. There were fewer bands in chloroplasts in mesophyll cells (2-7) than in those of cultured cells (7-16) (Fig. 6A), while there were more thylakoids in the chloroplast grana of mesophyll cells (5-9 in young leaves, 11-23 in old leaves) than in those of cultured cells (about 5 in PA and around 3 in PM cells) (Fig. 6B). The finding that chloroplasts in cultured cells had fewer thylakoids per granum and more bands of intergrana thylakoids than those in green leaves would reflect the lower Chl content of cultured cells, which allows the sufficient absorption of light energy.

Plastoglobuli and envelope invagination: One striking feature of chloroplasts in cultured cells was the presence of numerous small plastoglobuli, especially in PA cells (Fig. 3). Figs. 2B and C show that the size and distribution of large plastoglobuli in the chloroplasts of a mature leaf and an old leaf differed: those in mesophyll cells were large and few, while those in cultured cells were small and numerous. Moreover, many invaginations of the inner envelope into the stroma and vesicles near the envelope were observed in the chloroplasts of cultured cells during chloroplast division (Figs. 3 and 4).

*Changes in the activities of photochemical reactions during growth*

Hill reaction (PS II + PS I) and PS I activities were determined for cultured and mesophyll cells of tobacco as a function of the Chl content (Fig.7). In PA cells, these photochemical activities were nearly constant (ca. 70-80 μmol $O_2$ evolution/mgChl/h for the Hill reaction and ca. 140-160 μmol $O_2$ consumption/mgChl/h for PS I activity) through the 3rd week of incubation, after which they decreased. These values were about half of the maximum activities of green leaf cells.

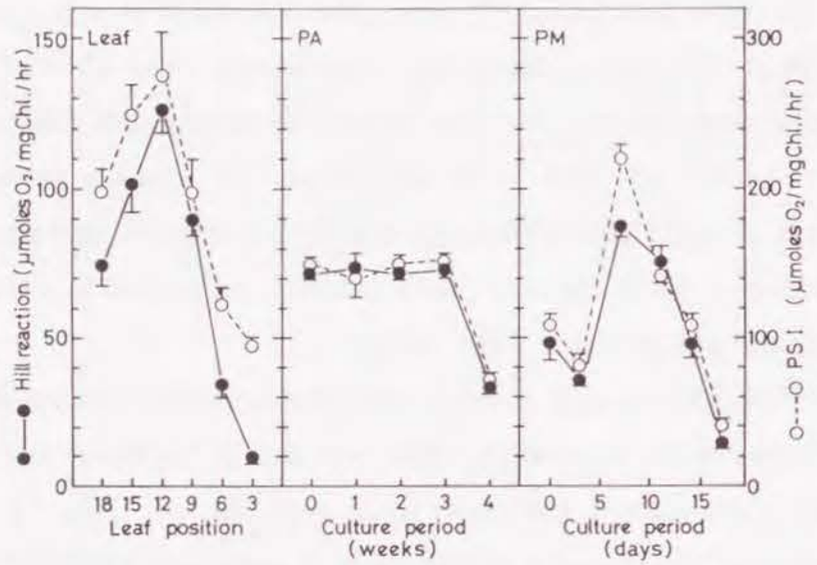



Fig. 7. Changes in photosynthetic activities during the growth of leaves and cultured cells of tobacco. Vertical bars indicate the standard error of the activities measured.

The pattern of change for PM cells differed from the patterns for the PA and green leaf cells. Both the Hill reaction and PS I activities in the PM cells were highest during the early



exponential phase (7 days after inoculation), at which time they were similar to the values for PA cells. Thereafter, they showed a continuous decrease during PM cell growth. These changes in the photochemical activities of the PA and PM cells were not correlated with changes in their Chl contents. In contrast, the changes in the photochemical reaction activities of the mesophyll cells coincided with changes in the Chl content per unit fresh weight during leaf development.

*Analyses of thylakoid membrane polypeptides*

As described above, the photosynthetic activities of cultured cells on a per mg Chl basis indicate that chloroplasts of cultured green cells are poorly developed, even in cells that grow photoautotrophically. Thylakoid membrane polypeptides were assayed by LDS-PAGE to investigate the lower photosynthetic activity of chloroplasts in cultured cells at the molecular level. For this analysis, the chloroplast preparations were further purified to eliminate contamination by other organelles, such as mitochondria. Chloroform/methanol extraction was used to separate the integral membrane proteins from the peripheral ones. The heat dissociation of pigment-protein complexes and other characteristics reported in the literature were used to identify the polypeptides present (CF1: Harak and Hill 1972, PS I /PS II: Mulett et al. 1980).

The thylakoid membrane polypeptides from both types of cultured cells and from mesophyll cells are shown in Fig. 8. Each lane was loaded with an equal amount of chlorophyll. The cultured and mesophyll cells had similar thylakoid compositions with and without heat dissociation. On the other hand, there were

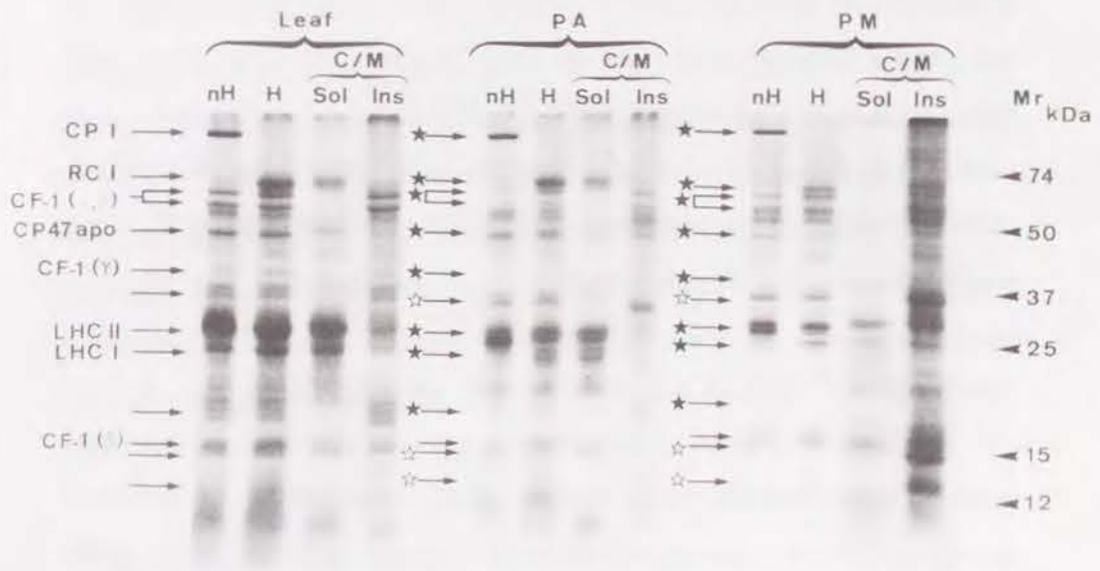



Fig. 8. Comparison of thylakoid membrane polypeptides of leaves and cultured cells. Chloroplasts were isolated from mature leaves, from PA cells after 2 weeks of incubation and from PM cells after 7 days of incubation. Polypeptides which decreased or increased in amount are indicated by a black star or a white star, respectively. nH: nonheated membrane samples. H: membraned samples heated (100°C, 1 min). C/M-sol: membrane samples soluble in a 2:1 mixture of chloroform/methanol. C/M-Ins: membrane samples insoluble in chloroform/methanol. Each sample contained thylakoid membranes equivalent to 10 mg chlorophyll. CF-1: coupling factor. CP I: chlorophyll complex of PS I. CP47apo: apoprotein of the PS II reaction center. LHC I and LHC II: respective light harvesting complexes of PS I and PS II. RC I and RC II: respective reaction center polypeptides of PS I and PS II.



considerable reductions in the polypeptide contents on a Chl basis for both the soluble and insoluble fractions of the chloroform/methanol extracts of the cultured cells. The chloroform/methanol soluble fraction of the PM cells was particularly deficient in thylakoid polypeptides. The α-and β-subunits of CF1, LHC(I), and LHC(II), the polypeptides of the reaction center of PS I, and the 47 kD apoprotein of the PS II reaction center were all present in low amounts in the cultured cells. Certain polypeptides, mainly those present in the chloroform/methanol insoluble fraction, showed increases in the cultured cells (including those with molecular masses of 36 kD, 14 kD, and 13 kD).

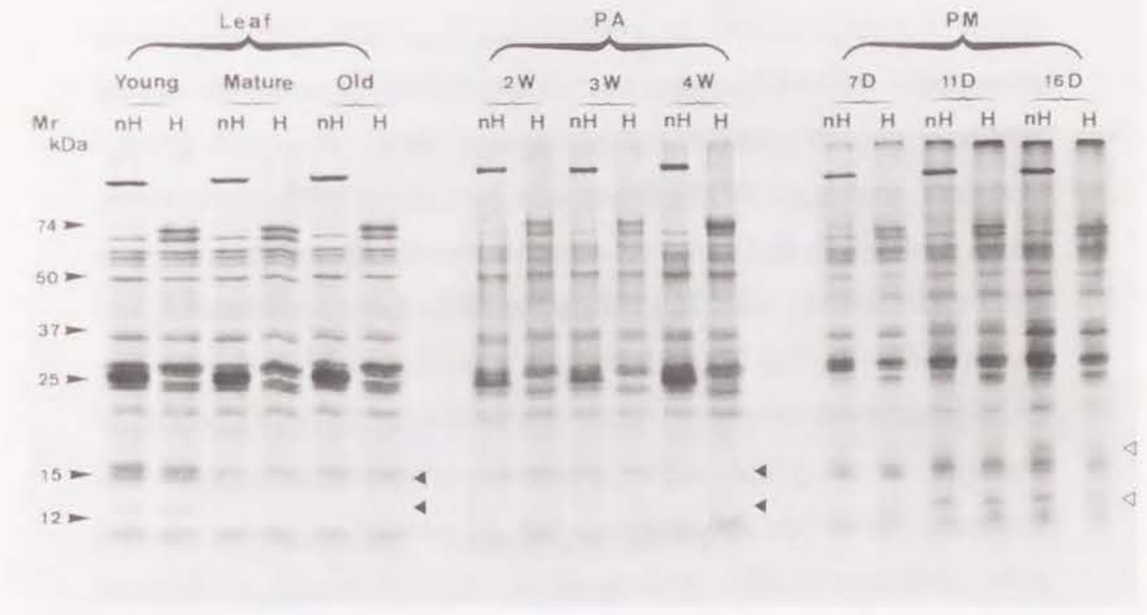



Fig. 9. Changes in thylakoid membrane polypeptides during the growth of leaves and cultured cells. Each slot contained heated (H) and nonheated (nH) thylakoid membranes equivalent to 10 μg chlorophyll. White and black triangles indicate polypeptides that increased and decreased during growth, respectively.

Photosynthetic activities changed with growth in all of the chlorophyllous cell types. However, LDS-PAGE analyses showed that the composition of the thylakoid polypeptides did not change significantly (Fig. 9). The amounts of the 16 kD and 12 kD polypeptides increased only slightly during the growth of PM cells. The amounts of the 15 kD and 13 kD polypeptides of the mesophyll and PA cells decreased slightly with growth. However, neither the increases nor the decreases in the amounts of thylakoid polypeptides were as remarkable as the changes in photosynthetic activities.

## DISCUSSION

There have been only a few reports of cultured green cells with Chl contents, on a fresh weight basis, comparable to those of mesophyll cells, although several cultures that grow without the addition of an organic carbon source have been established (Yamada and Sato 1978, Hüsemann and Barz 1979, Horn et al. 1983, La Rosa et al. 1984). The present study shows that there is little difference in the Chl content per chloroplast in cultured and mesophyll cells (Fig.1C) and that, on a fresh weight basis, the low chloroplast number per cell does not cause the low Chl content of these tobacco cells. The present results suggest that the difference between the cell volumes of mesophyll and cultured cells determines the difference in Chl content. Several researchers have shown that the average chloroplast number per cell increases with cell volume during leaf development (Possingham and Saurer 1969, Kameya 1972, Ellis and Leech 1985, and Fig.1C). Therefore, chloroplast proliferation may be suppressed in cultured cells by some regulatory mechanism, or



the increase in cell volume may exceed the increase in chloroplast number.

Except for the number of chloroplasts per cell, several differences between cultured cells and mesophyll cells were found by electron microscopy. One difference was the abundance of dividing chloroplasts in cultured cells, as comparison with mesophyll cells. Although the number of chloroplasts per cell increased during leaf development (Fig.1C) and chloroplast division was expected even in leaves, division was not observed in the limited number of observations of this study using one stage of young developing leaves (Fig.2A). This specific feature is derived from the nature of cultured cells, which are constantly growing. Cultured cells are thus believed to provide a new system for the study of the biogenesis of chloroplasts. Invagination of the inner envelope into the stroma and vesicles near the envelope during chloroplast division might be involved in the generation of new inner membrane systems in daughter chloroplasts.

Many plastoglobuli were observed throughout the growth cycle in cultured cells. Accumulation of plastoglobuli has been reported in senescing leaves, virus-infected leaves and ethylene-treated leaves (Toyama 1980). In fact, the accumulation of ethylene was observed in the present cell culture and the accumulation of plastoglobuli may reflect the stress condition induced in this *in vitro* cultured system, as well as accumulation of stress proteins (Chapter III).

Analyses of the development of chloroplast inner membrane systems showed that the chloroplasts in cultured cells have more bands of intergrana thylakoids and fewer thylakoids per granum than the chloroplasts in mesophyll cells. Among the cultured cells,

the mean number of thylakoids per granum in PA cells (5.17) was significantly higher than that in PM cells (3.10) (significant difference was analyzed by a t-test at P=0.01). Therefore, the chloroplasts in PA cells have more highly developed inner membrane systems than those in PM cells. Moreover, PM cells have chloroplasts in various developmental stages and the average number of intergrana thylakoids changed greatly during growth (from 7 to 16).

Measurements of photochemical reactions have also shown differences between the photosynthetic characteristics of cultured green cells and mesophyll cells. In green leaves, on a fresh weight basis, these activities change concomitantly with changes in the Chl content, as reported for wheat (Camp et al. 1982) and soybean leaves (Ford and Shibles 1988). In cultured cells, changes in photochemical reactions do not follow those in Chl content.

Photosynthetic activities in PM cells also decrease during the lag phase, and then increase to reach a maximum in the early exponential phase (7 days after inoculation). On the other hand, PA cells show relatively constant activity during growth (through the 3rd week). The initial decrease seen for PM cells may be caused by the high concentration of sucrose in fresh medium (La Rosa et al. 1984). However, after 7 days of incubation (in the early exponential phase), when the concentration of sucrose is still high (data not shown), the photosynthetic activities of PM cells reach maximums. One possible explanation for this increase in photosynthetic activity is the rapid supply of $CO_2$ which is evolved by the high rate of respiration in the early exponential phase (Sato et al. 1979). One Possible explanation for the rapid



decrease in activity is that this deterioration of photosynthetic competence may be due to the depletion of nutrients in the growth medium, especially under photomixotrophic conditions because cell density increases rapidly and to a greater extent than it does for PA cells.

Analyses of thylakoid membrane polypeptides show that chloroplasts of cultured cells have polypeptide compositions qualitatively similar to those of mesophyll cells even though the amounts of some polypeptides are lower in the cultured cells (particularly in PM cells). The ratio of uncoupled to coupled electron transport activity in isolated thylakoids indicates that the reduced coupling in thylakoids of cultured green cells (uncoupled/coupled=1-2.6 in PM cells, 2-3.4 in PA cells and 3.3-6.5 in green leaves) is correlated with the loss of CF1. Therefore, the decrease in some thylakoid polypeptides (i.e., the polypeptides of CF1) may have a greater effect on lowering photosynthetic activities in cultured cells than they do in mesophyll cells.

The amounts of certain polypeptides increased in the thylakoid membranes of this cultured cells. Due to the difficulty of purifying the chloroplasts of cultured green cells, the subcellular fraction of heterotrophically cultured cells was isolated to determine whether the polypeptides which increased in these thylakoid preparations of cultured green cells are of chloroplast origin. Analyses of the polypeptides of mitochondrial, nuclear and cell wall fractions indicated that these polypeptides do originate in the chloroplast (data not shown). Whether they function as regulatory factors in chloroplast biogenesis, or are degraded products of large components of thylakoid polypeptides or stress proteins would be an interesting subject for future study.

Cultured green tobacco cells have PS I/PS II activity ratios similar to those of green leaves, although their actual activities are lower. Furthermore, cultured cells have a polypeptide composition which is qualitatively similar to that of the thylakoid membrane of mesophyll cells. These results suggest that overall chloroplast gene expression may be suppressed in cultured green cells. This decrease in gene expression may be directly or indirectly affected by the stresses which are reflected by the accumulation of plastoglobuli in chloroplasts in cultured cells and stress proteins.



CHAPTER II

# EFFECTS OF SEVERAL HERBICIDES ON PHOTOAUTOTROPHIC, PHOTOMIXOTROPHIC AND HETEROTROPHIC CULTURED TOBACCO CELLS AND SEEDLINGS

In chapter I, I investigated the development of chloroplasts in PA cultured cells and photosynthetic activities in comparison with mesophyll cells and concluded that PA cells have many similarity as mesophyll cells beside several differences. To get more information about the functional similarity of cultured cells and mesophyll cells, I used several herbicides as chemicals to characterize the plant cell responses.

Herbicides have been developed to control weeds due to their selective action on the plant metabolism. The mode of action of several herbicides is known to be specific to certain metabolic processes. Based on this knowledge, I compared the response of both cultured cells and seedlings to a wide spectrum of herbicides. The 12 herbicides used in this study are thought to have different primary mode of action (Ashton and Crafts 1981, Dodge 1983). Atrazine and DCMU primarily affect the photosynthetic electron transport in chloroplasts. Propanil inhibits photosynthesis, but an inhibitory effect on root growth was also reported. Paraquat diverts part of the electron flow in chloroplasts by the competition with ferredoxin for electrons from with oxygen. Nitrofen is thought to be activated in the light and inhibit electron transport and photophosphorylation. 2,4-D is postulated to disturb the auxin action in plant cells although the exact mode of action still

remains to be elucidated. Diphenamid is phytotoxic mainly, as a result of the inhibition of cell division. Glyphosate inhibits 5-*enol*pyruvyl shikimate-3-phosphate synthase, one of the key enzyme in the biosynthesis of aromatic amino acids (Amrhein et al. 1980). Bialaphos, an antibiotic produced by *Streptomyces hyroscopicus* SF-1293 (Kondo et al. 1973), is used as a nonselective contact action-type herbicide in Japan. Its catabolic product (phosphinothricin) inhibits glutamine synthetase, a key enzyme of nitrogen assimilation (Leason et al. 1982). DNBP is an uncoupler which inhibits respiration as well as photosynthesis. Sodium chlorate is a strong oxdizer. The primary mode of action of DTP is postulated to be the inhibition of chlorophyll biosynthesis (Kawakubo et al. 1979).

While heterotrophically cultured cells have been used to investigate the effects of some herbicides or to select herbicide resistant cells or plants (Zilkah et al. 1977, Gressel et al. 1978, Davis and Shimabukuro 1980, Nafziger et al. 1984, Chaleff and Ray 1984), the photosyntheis-inhibiting herbicides are not particularly effective in inhibiting the growth of these cells with low photosynthetic rates. However, Cséplö and Medgyesy (1986) were successful in observing the effect of photosynthesis-inhibiting herbicides in photomixotrophic cultured tobacco cells when they were grown in medium with a low sucrose concentration. The report on photoautotrophic cells was limited to atrazine (Horn and Widholm 1984). The present study compares the response of tobacco seedlings and photoautotrophically cultured tobacco cells to 12 herbicides with different mode of action, and discusses the possibility of using PA



cells as a model system to investigate the effects of herbicides in higher plants.

## MATERIALS AND METHODS

### *Chemicals*

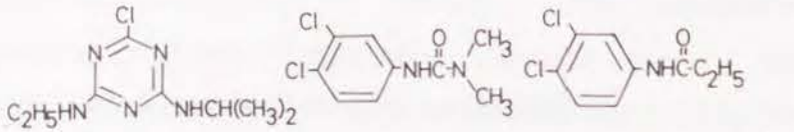
The herbicides examined were atrazine, glyphosate, bialaphos and DTP (kind gifts from Ciba-Geigy, Monsanto, Meiji Seika Co. Ltd. and Sankyo Co. Ltd., respectively), propanil, nitrofen and diphenamid (kindly provided by Sumitomo Chemicals Co. Ltd. as analytical reagents), DCMU, paraquat, DNBP and 2,4-D (purchased as analytical reagents, except that DCMU was further purified by the recrystallization from benzene/ethylacetate solution before use). Their structures are shown in Fig. 1.

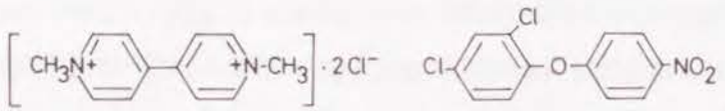
### *Cultured cells*

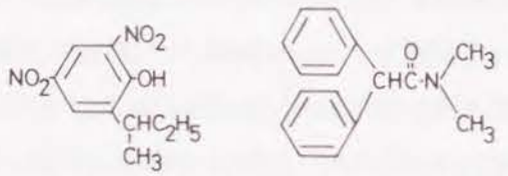
Photoautotrophic and photomixotrophic cells were cultured as described in chapter I. Heterotrophic cells were cultured in the same media as photomixotrophic cells in the dark.

### *Seedlings*

Tobacco (*Nicotiana tabacum* cv. Samsun) seeds were surface-sterilized by sequential treatment with 70% ethanol for 30 s, 0.2% benzalkonium chloride for 15 min. and 2% sodium hypochloride for 15 min. After washing with sterile water, seeds were germinated on water-moistened sterile filter papers. Healthy seedlings were utilized for the experiments about two weeks after germination.



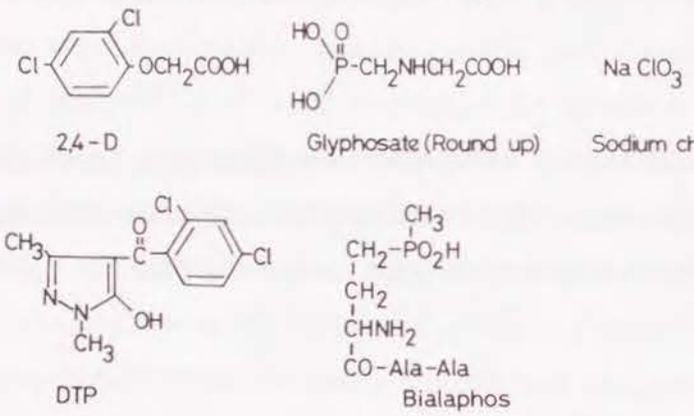




Fig. 1. Structure of the herbicides used.

*Test of herbicide toxicity*

Herbicides in methanol or in filter-sterilized aqueous solution were added to culture medium at final concentration of 0.01 mM - 1 mM. Fresh weight of 0.5 g of cultured cells was inoculated in 12.5 ml of culture medium in 50 ml flasks. Photoautotrophic



cultures were in a chamber in which the $CO_2$ concentration was kept at about 1% by flushing with $CO_2$ enriched air (Fig. 2). In this photoautotrophic culture, silicone sponge cap (Silico-sen®, Shin-Etsu-Polymer Co. Ltd., Japan) was used to facilitate the gas exchange. Cultured cells were harvested after 2 weeks (photomixotrophic cells) or 3 weeks (photoautotrophic and heterotrophic cells) of incubation when the controls reached early stationary phase. The average increase in fresh weight of photoautotrophic, photomixotrophic and heterotrophic cells at harvest was 1.0 g, 5.5 g, 5.0 g, respectively. The effect of the herbicides on the cultured cells was evaluated by determining the increase in fresh weight of control and herbicide-treated cells and calculating relative growth where; Relative growth = (Increase in fresh weight of cells treated with herbicide / Increase in fresh weight of control cells) x 100 (%).

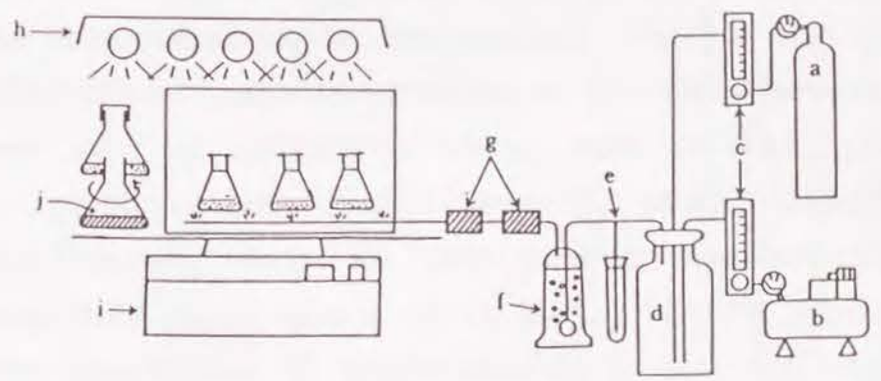



Fig. 2. System for photoautotrophic culture of tobacco cells consisting of a) $CO_2$ gas, b) air compressor, c) flow control, d) reservoir for mixed gas, e) safety valve, f) distilled water to humidify and wash gas, air line filter to keep sterile condition, h) illumination, i) shaker, j) carbonate buffer.

The effects of herbicides were examined with seedlings grown on moistened filter papers in petri-dishes. The filter paper contained ten ml of half strength Linsmaier and Skoog basal medium with different concentrations of herbicides. The effect of the herbicides was evaluated semi-quantitatively using the following scale: Healthy, green seedlings = 2, pale green seedlings = 1. Seedlings showing chlorosis or necrosis = 0. The total score of the 20 seedlings used for each treatment was summed and the relative effect was calculated as follows: Relative effect = (Total score for 20 herbicides-treated seedlings / Total score for 20 control seedlings) x 100 (%).

## RESULTS AND DISCUSSION

Photoautotrophic, photomixotrophic and heterotrophic cells showed very different responses to the different classes of herbicides used (Figs. 3 and 4). Herbicides which inhibit photosynthetic process suppressed the growth of photoautotrophic cells most strongly, as compared to photomixotrophic and heterotrophic cells (Fig. 3). When the $I_{50}$ value ( $I_{50}$ = herbicide concentration at which growth is inhibited by 50%) were compared among cultured cells, the sensitivity of photoautotrophic cultured cells for these photosynthesis-inhibiting herbicides, was on the average 60 or 2,500 times higher than that of photomixotrophic or heterotrophic cells, respectively.

Interestingly, the photomixotrophic cells were also sensitive to photosynthesis inhibitors when grown in medium with a high (3%) sucrose concentration, although the photoautotrophic cells were the most sensitive to these herbicides. Cséplö and



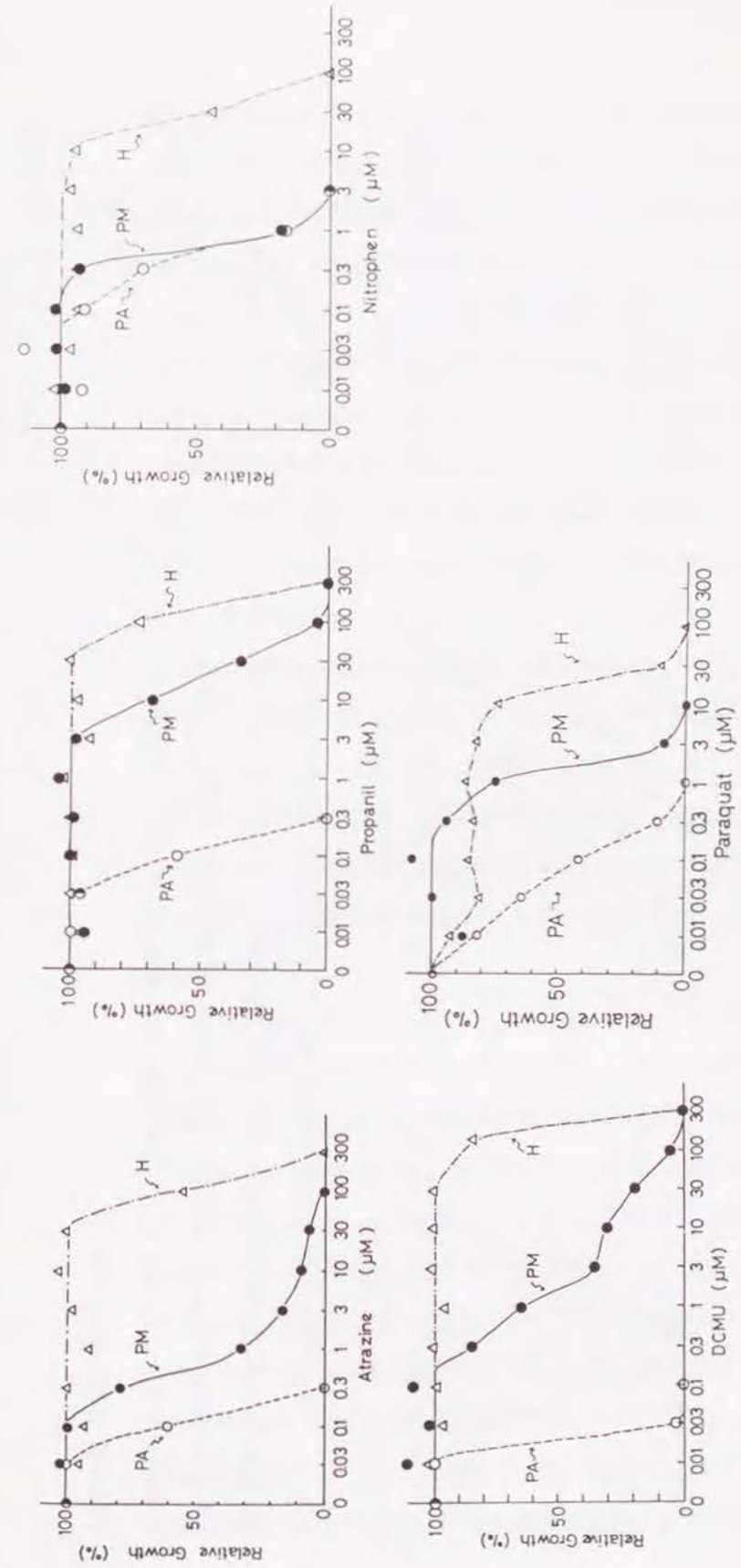



Fig. 3. The effects of herbicides known to inhibit photosynthesis (atrazine, DCMU, propanil, paraquat and nitrofen) on the growth of photoautotrophically (PA), photomixotrophically (PM), and heterotrophically (H) cultured cells and seedlings (S).
○: Photoautotrophic cells, ●: Photomixotrophic cells,
△: Heterotrophic cells, ■: Seedlings

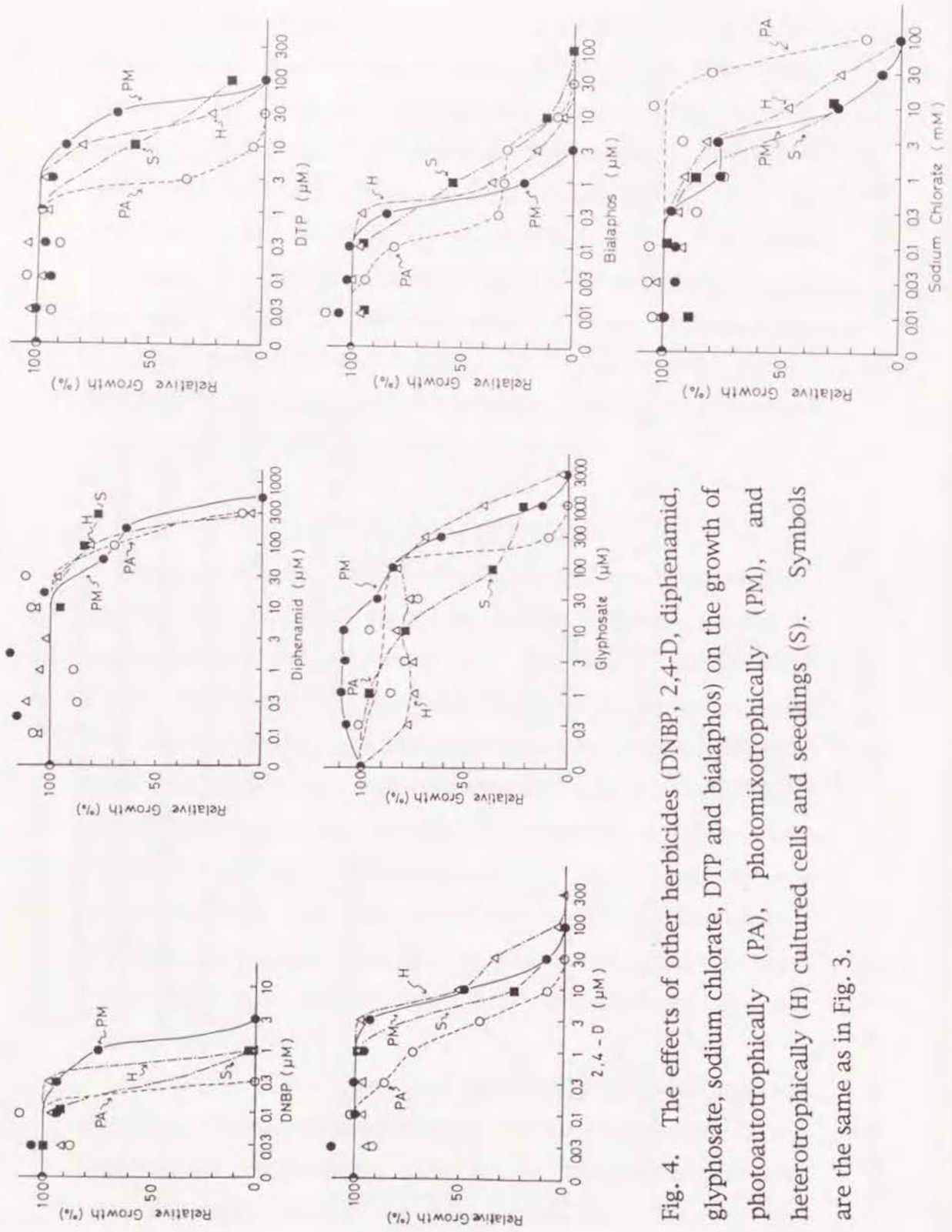



Fig. 4. The effects of other herbicides (DNBP, 2,4-D, diphenamid, glyphosate, sodium chlorate, DTP and bialaphos) on the growth of photoautotrophically (PA), photomixotrophically (PM), and heterotrophically (H) cultured cells and seedlings (S). Symbols are the same as in Fig. 3.



Medgyesy (1986) reported that their cultured green cells did not respond to photosynthesis-inhibiting herbicides at 3% sucrose, while the herbicide toxicities were greatly enhanced by reducing the sucrose concentration to 0.3%. These different responses of cultured cells could be due to the different photosynthetic potentials of cultured cell lines used. The photomixotrophic cells used in this study, cultured at 3% sucrose, showed not only considerable photosynthetic activities (50-100 μmoles$O_2$ evolution/mg Chl/h) but light also enhanced their growth, even under the liquid culture condition (Chapter I).

Herbicides which have modes of action other than photosynthesis inhibition, suppressed the growth of all types of cultured cells at similar concentrations (Fig. 4). While differences in sensitivity among various types of cultured cells was small for these herbicides, photoautotrophic cells were still the most sensitive. Sodium chlorate was an exception to this, however and the photoautotrophic cells were the least sensitive to this herbicides.

When the herbicide sensitivities of the cultured cells are compared with those of seedlings, photoautotrophic cells showed the responses most similar to those of the seedlings (Figs. 3 and 4, Table 1). Statistical analyses (t-test) clearly showed that the $I_{50}$ of photoautotrophic cells ($r=0.72$, $P<0.01$) was more similar to the $I_{50}$ of seedlings than to the $I_{50}$ of photomixotrophic ($r=0.59$, $P<0.05$) and heterotrophic ($r=0.28$, $P>0.05$) cells (Fig. 5). While the correlation coefficients of photomixotrophic cells with seedlings and of heterotrophic cells were similar with the values of obtained by Gressel et al. (1978), the correlations of the responses of photoautotrophic cultured cells with those of seedlings obtained in

Table 1.

Effect of herbicides on the growth of photoautotrophically (PA), photomixotrophically (PM), heterotrophically (H) cultured cells and seedlings of tobacco

| Herbicide | $I_{50}$* (μM) PA | PM | H | S | $I_{50}$(H)/ $I_{50}$(PA) |
|---|---|---|---|---|---|
| Atrazine | 0.1 | 0.5 | 100 | 0.1 | 1000 |
| DCMU | 0.02 | 1.0 | 200 | 0.03 | 10000 |
| Propanil | 0.1 | 20 | 100 | 1.0 | 1000 |
| Paraquat | 0.07 | 2.0 | 20 | 0.4 | 300 |
| Nitrofen | 0.5 | 0.5 | 30 | 1.0 | 60 |
| Diphenamid | 200 | 300 | 200 | 300 | 1 |
| DNBP | 0.2 | 2.0 | 0.6 | 0.3 | 3 |
| 2,4-D | 2.0 | 10 | 10 | 5.0 | 5 |
| Glyphosate | 200 | 500 | 700 | 50 | 3.5 |
| NaClO3 | 60** | 6** | 9** | 3** | 0.15 |
| DTP | 3.0 | 40 | 20 | 10 | 6.7 |
| Bialaphos | 0.2 | 0.5 | 0.6 | 1.0 | 3 |

* $I_{50}$: The concentration in which half of the growth of cells were inhibited by the addition of herbicides tested
** mM

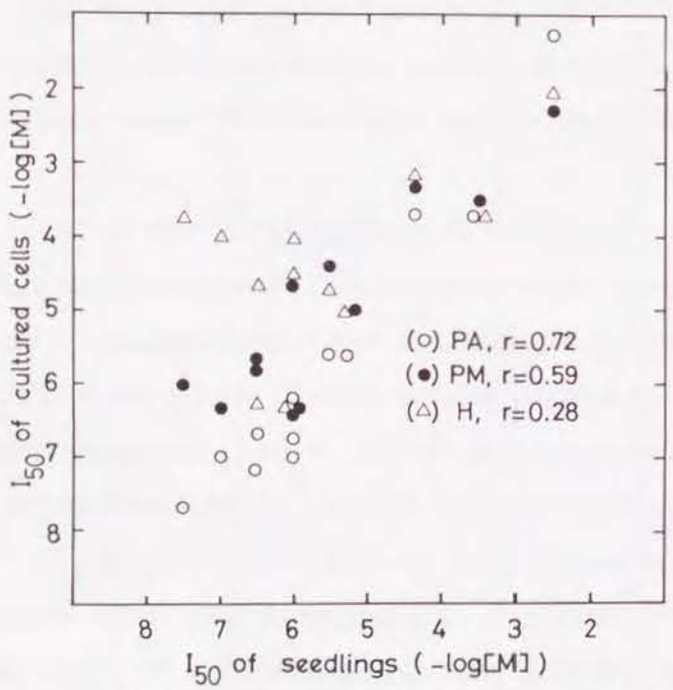



Fig. 5. Correlation between I50 of cultured cells and seedlings. Symbols are the same as in Fig. 3.



the present study are even higher than these. This high sensitivity of PA cells are now used for the survey of new herbicides which inhibit the photosynthetic electron transport (Sato et al. 1991).

However, a relatively low correlation between the I50 of photoautotrophic cells and seedlings was observed with diphenamid and glyphosate. These responses of cultured cells would reflect the different metabolic activities of cultured cells and intact plant cells; high cell division rate and lower production of secondary metabolizes derived from aromatic amino acids in the cultured cells might be one possible explanation for the differences in the responses to diphenamid and glyphosate, respectively. Less sensitivity for glyphosate also was reported with different cultured tobacco (Cséplö and Medgyesy 1986).

In general, cultured green cells, especially photoautotrophic cells, showed higher sensitivity to various herbicide than heterotrophic cells and were more similar to plants, as mentioned above. However, Gressel et al. (1978) reported that napronamide was more toxic to heterotrophic cells than green cells or intact plants. The combination of photoautotrophic cells and heterotrophic cells may thus provide us a more broad survey system to detect the phytotoxicity of herbicides.

Photoautotrophic cultured cells might be useful for investigations on the modes of action of herbicides. Propanil is known to inhibit the photosynthetic process but the site of its primary action is still obscure because it also has an inhibitory effect on root growth. However, when I examined the effects of propanil on the growth of various types of cultured cells, the

results indicated that propanil primarily inhibited photosynthesis (Fig. 3, Table 1).

DTP is reported to inhibit chlorophyll synthesis (Kawakubo et al. 1979). However, the results indicated that DTP inhibited not only chlorophyll synthesis, but also other metabolic reactions because heterotrophic cells were inhibited at similar concentrations as photoautotrophic cells (Fig. 4, Table 1). Further elucidation of the mode of action of this herbicides is required.

Photoautotrophically cultured cells might be the most useful for the selection of cells resistant to photosynthesis-inhibiting herbicides. Heterotrophically cultured cells have been used to select herbicide-resistant cells, but their applications were limited to herbicides having modes of action other than photosynthesis inhibition because photosynthesis-inhibiting herbicides showed little effects on heterotrophic cells, as described above. Photoautotrophic or photomixotrophic cells with active photosynthetic functions are required for such selection. Recently, Sato et al. established a new atrazine resistant tobacco cell line which showed cross-resistance to DCMU by cellular selection from the tobacco cells used in this study (Sato et al. 1988). It is no doubt that photoautotrophic cultured cells are one of the materials suitable for research on herbicides and other plant growth regulation substances.



CHAPTER III

# CHARACTERIZATION OF POLYPEPTIDES THAT ACCUMULATE IN CULTURED TOBACCO CELLS

In the previous chapters, I reported the basic properties of photoautotrophically cultured cells of tobacco. In this chapter, I will report the more detailed characteristics of gene expression in cultured cells. Cultured plant cells have been used both for biochemical and physiological studies, and to produce useful secondary metabolites (Green et al. 1987). The metabolism of cultured cells, however, often differs from that of intact plant cells, and little is known about the mechanism that controls their metabolic differentiation.

Photoautotrophically cultured cells of *Nicotiana tabacum* are useful as a model system to investigate the regulation of gene expression in cultured cells because photoautotrophically cultured cells which grow without an organic carbon source have a metabolic differentiation (i.e. photosynthetic functions) very similar to that of mesophyll cells from green leaves, even though the development and activity of the chloroplasts are limited and $CO_2$ enriched air is needed for growth (Hüsemann et al. 1979, Yamada et al. 1982, Horn and Widhlom 1984 and also Chapter I and II). This functional similarity and the availability of relatively homogeneous cells facilitate characterization of the specificity of each cell type. I therefore characterized the polypeptides that accumulate in photoautotrophically cultured green tobacco cells, and in mesophyll tobacco cells as well as other cultured cells grown under different culture conditions to

determine what mechanism controls gene expression in cultured cells. The analysis by 2D-PAGE and microsequencing of polypeptides clearly show that in vitro cultured cells accumulate stress proteins which were also found in old leaves, roots and leaves infected with Tobacco Mosaic Virus, but not in young healthy leaves.

## MATERIALS AND METHODS

### *Plant and cell materials*

Photoautotrophic, photomixotrophic cells of tobacco (NI line) were cultured as described in Chapter I. Heterotrophic cells (NI line) were maintained as described in Chapter II. A new cell line (NII line) that retained its ability to regenerate but showed little chloroplast development was induced on modified Linsmaier-Skoog medium containing 10 μM naphthaleneacetic acid and 1 μM kinetin. Adventitious shoots were induced from this NII line on the same medium containing 0.1 μM naphthaleneacetic acid and 10 μM benzyladenine. All cultured cells were grown in liquid media on rotary or reciprocal shakers at 100 rpm and 25±2°C in light, except heterotrophic cells which were grown in the dark. Intact plants of tobacco were grown for 2-3 months in soil in a greenhouse under natural illumination.

### *Extraction of total cellular protein*

The total cellular protein present in the cultured and mesophyll cells was extracted according to Schuster and Davies (1983). One gram of cells was frozen in liquid nitrogen then ground with a pestle to a fine powder in a precooled mortar. After the temperature reached ca. 0°C, 15 ml of ice-cold



extraction buffer (0.7 M sucrose; 0.5 M Tris; 30 mM HCl; 50 mM EDTA; 0.1 M KCl; 2% (v/v) mercaptoethanol) was added together with 0.1 g of polyvinylpyrrolidone, and the homogenate was filtered through Miracloth. Proteins were extracted from the filtered solution with an equal volume of phenol saturated with water then precipitated by mixing them with 0.1 M ammonium acetate in methanol. The precipitate was air dried and redissolved in the lysis buffer (9.5 M urea; 2% Nonidet P-40; 2% ampholines (pH 3.5-10 : pH 9-11 : pH 2-4 = 16:3:1); 5% mercaptoehanol).

Protein was measured by the Coomassie-blue protein quantification method described by Bradford (1976) and compared to values for Bovine serum albumin standards, which also had been dissolved in the lysis buffer. The final protein concentration was adjusted to 10 mg/ml.

### *2D-PAGE and protein electroblotting on PVDF membranes*

Two-dimensional gel elctrophoresis was done with slight modifications, according to the method of O'Farrell (1975). A 400 μg sample of protein was loaded for the first dimension. Molecular weight markers (Amersham) that ranged from 14,300 to 200,000 were used to determine the molecular weights of the polypeptide spots. The pH range of the isoelectric focusing gels was obtained by cutting each gel into 5 mm slices which I soaked in vials containing one ml of degassed, distilled water for 1 h prior to measuring the pH.

Protein electroblotting on PVDF membranes was done as described elsewhere (Bauw et al. 1987, Matsudaira 1987). The gels first were equilibrated for ca. 30 min in transfer buffer

composed of 500 mM boric acid, 500 mM Tris and 10% methanol. The electroblotting used a transfer and was done with an LKB NOVA blot apparatus set at a constant 198 mA for 3 h; no SDS was added to the transfer buffer. Immobilized proteins were made visible with Coomassie blue. The spots were cut off with scissors and stored at -20 °C in eppendorf tubes.

*Microsequencing of the $NH_2$-terminal amino acid residue*

The PVDF membranes which bound the proteins were cut into small pieces (1mm x 1 mm) that were placed on a glass filter that had been treated with Polybrene. They were immediately subjected to Edman degradation in order to sequence the $NH_2$-terminal amino acid. An Applied Biosystem (Model 477A) sequencer equipped with an on-line PTH-analyzer that ran the regular sequence program was used.

## RESULTS AND DISCUSSION

*2D-PAGE analysis of the total cellular extract of photoautotrophically cultured cells and mesophyll cells*

Analysis of the total cellular proteins by SDS-PAGE showed that the polypeptides of photoautotrophically cultured tobacco cells differed in several respects from those of mesophyll cells (Fig. 1); therefore, I separated the total cellular polypeptides by 2D-PAGE analyses as described in "MATERIALS AND METHODS".

Two-dimensional PAGE analyses showed that photoautotrophically cultured cells accumulate polypeptides that differ considerably from those accumulated by mesophyll cells (Fig. 2, Table 1), although the former cells grow only by



photosynthesis and have photosynthetic functions and a structure similar to those of mesophyll cells. Protein L1-L7 were present relatively larger amounts in mesophyll than in cultured cells; whereas, in cultured cells the amounts of proteins P1-P14 (with high pIs) were relatively larger.

Proteins L1 and L1' were identical to the large subunit of ribulose 1,5-bisphosphate carboxylase (Rubisco) and L2 and P15 to the small subunit of Rubisco when immunoblotted with specific antiserum prepared against tobacco Rubisco.

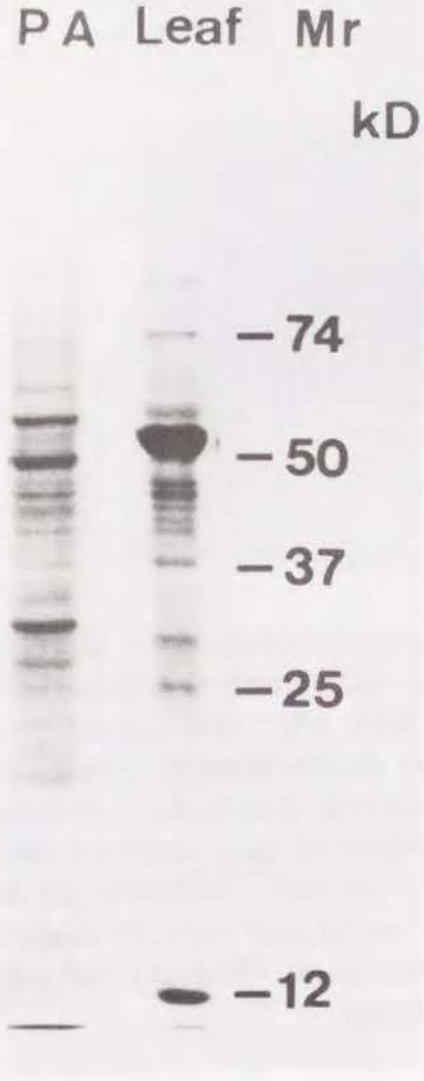



Fig. 1. SDS-PAGE of the total cellular proteins of photoautotrophically cultured cells and leaves. Proteins were extracted from photoautotrophically cultured cells (PA; 1 week after inoculation) and half expanded leaves (Leaf) with extraction buffer then desalted through small sephadex G25 column. A 10 μg protein sample mixed with SDS-loading buffer which had been denatured at 100°C for 2 min was layered on 12% polyacrylamide gel. Mr indicates the molecular weight of proteins.

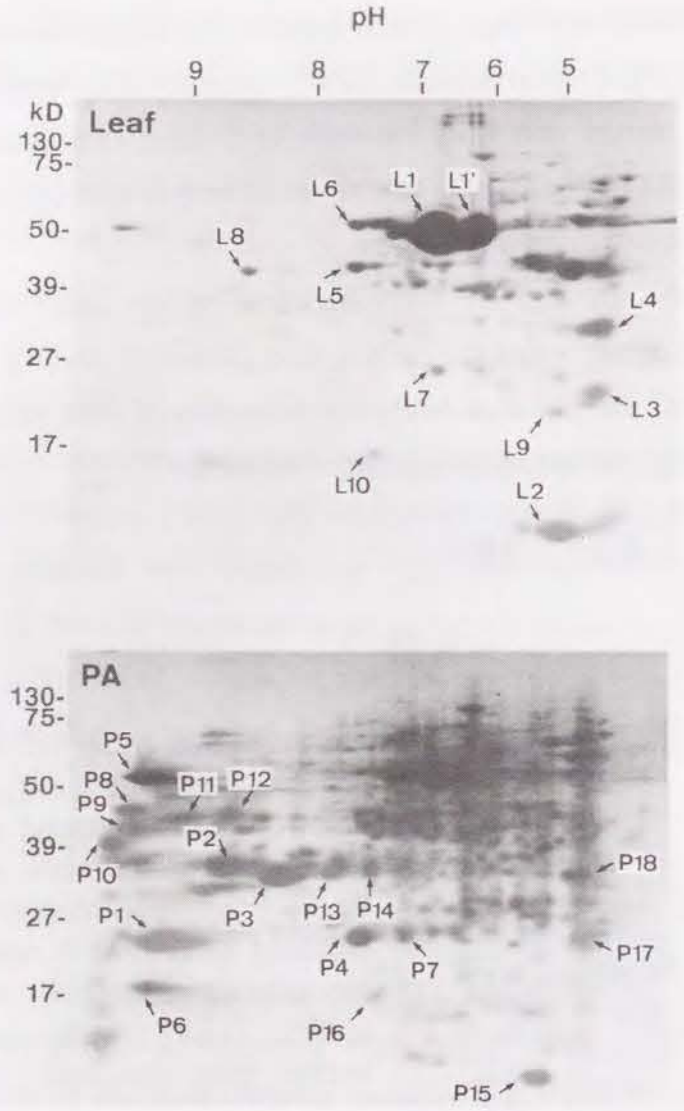



Fig. 2. PVDF membrane blots of proteins separated by 2D-PAGE. Total cellular protein extracts from half expanded leaves (Leaf) and photoautotrophic cells(PA; 1 week after inoculation) were separated by equilibrium pH gel electrophoresis (horizontal direction) followed by SDS-PAGE (vertical direction). Proteins were electroblotted on PVDF membranes and detected with Coomassie blue. The pH values for the first dimension gel are given at the top and the Molecular weight (Mr) markers used in the second dimension gel at left of the figure. Proteins that were most abundant in each cell are numbered.



**Table 1** $NH_2$-terminal amino acid sequences of polypeptides that accumulate in cultured green cells (P1–17) and green leaves (L1–10) of tobacco

| Proteins | MW (kD) | pI | Presence in leaf/PA cells | $NH_2$-amino acid sequence | Protein expected |
|---|---|---|---|---|---|
| L1 | 55 | 6.8 | ++++/+ | blocked | Rubisco L |
| L1' | 55 | 6.4 | ++++/+? | blocked | Rubisco L |
| L2 (=P15) | 12 | 5.0 | +++/+ | XQVWPP(Y/I)(G/N)KKKYETLSYLPDLSQEQLLSE | Rubisco S |
| L3 (=P17) | 23 | 4.8 | ++/+ | AYGEAANVFGKP | EM23kD |
| L4 (=P18) | 33 | 4.8 | ++/+ | EGVPXRLT(Y/F) | |
| L5 | 42 | 7.6 | ++/+? | not analyzed | |
| L6 | 58 | 7.6 | ++/+? | blocked | |
| L7 | 27 | 6.8 | +/? | ELQSXXGA(K/A)PFI(S/Y) | |
| L10 | 17 | 7.3 | +/+ | AVQAAEVQ(D/S)XVTXXVYFDIS | |
| P1 | 26 | 10 | −/+++ | ATIEVRNNXPYTVWAASTPIGGGRRLDRGQTXVINAP | Osmotin 1 |
| P2 | 33 | 8.6 | −/+++ | EQXGSQAGGARXPSGLXXSKFGXXGNTNDYXGPGN | Chitinase |
| P3 | 32 | 8.2 | −/+++ | blocked | |
| P4 | 26 | 7.5 | −/++ | SGVFEVHNNXPYTVWAAATPVGGGRRLEXGQSWXFXAPP | Osmotin? |
| P5 | 55 | 10 | −/++ | blocked | |
| P6 | 18 | 10 | −/++ | blocked | |
| P7 | 26 | 7.0 | −/+ | SGVFEVHNNXPYTVWAXATPVG | Osmotin? |
| P15 (=L2) | 12 | 5.0 | +++/+ | XQVWPP(Y/I)(G/N)KKKYETLSYLPDLSQEQLLS | Rubisco S |
| P17 (=L3) | 23 | 4.8 | ++/+ | AYGEAANVFGKPXT | EM23kD |
| P18 (=L4) | 33 | 4.8 | ++/+ | EGVPXRLTXFDEI | |

Presence of each polypeptides were determined visually. Rubisco L and S: Large and small subunits of Rubisco. EM23kD: 23 kD extrinsic membrane protein in the water-splitting complex. Osmotin?: isoform of osmotin.

*Microsequence analyses of polypeptides that accumulate in cultured green cells*

I sequenced the major tobacco cell proteins in order to identify the kinds of protein that accumulated in photoautotrophically cultured and mesophyll cells. In the sequencing, L2-L4, L6, L7, L10, P1, P2, P4, P7 and P15-P17 spots could be sequenced for 9-39 cycles (Table 1).

All the sequences obtained were compared with protein sequences in the National Biomedical Research Foundation Protein Sequence Data Bank. There was significant homology between the $NH_2$-terminal sequences of four of the *Nicotiana* proteins and the corresponding region of the proteins reported previously (Muller et al. 1983, Vater et al. 1986, Shinshi et al. 1987, Singh et al. 1987a, Fig. 3): (i) The $NH_2$-terminal amino acid sequence of osmotin (Singh et al. 1987a), except for 3 residues which I could not identify. The sequence containing the residues of P4 and P7 also showed high homology to the $NH_2$-terminal of osmotin. The tobacco pathogenesis-related protein (PR-S; Cornelissen et al. 1986, Payne et al. 1988, van Kan et al. 1989) also showed high homology to P1, P4 and P7, but the degree was less than for osmotin. (ii) The sequence containing residues of P2 showed very high homologies to the $NH_2$-terminal of tobacco chitinase (Shinshi et al. 1987). (iii) L2 and P15 showed high homologies to the $NH_2$-terminal of the tobacco Rubisco small subunit (Muller et al. 1983). (iv) L3 and P17 showed high homologies to the $NH_2$-terminal of extrinsic membrane proteins in the water-splitting complex of spinach (Vater et al. 1986).



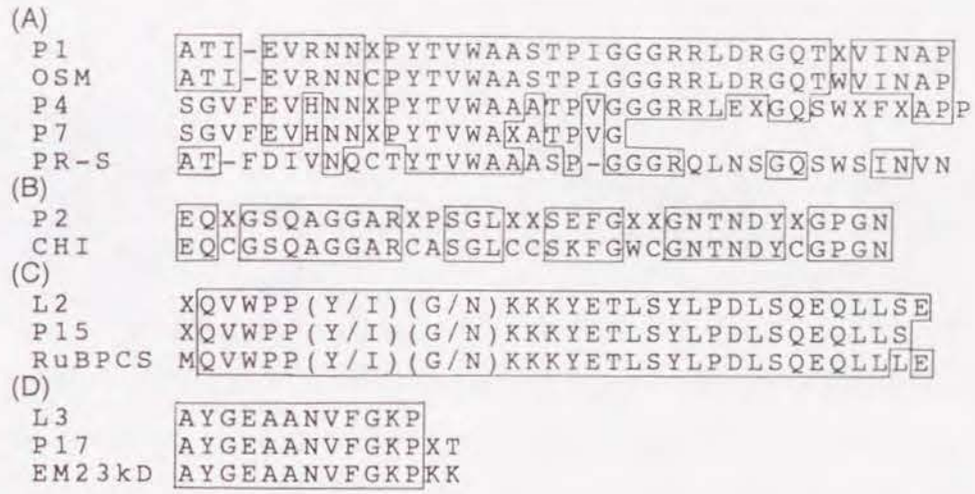



Fig. 3. Amino acid sequence alignments of (A): protein P1 (P1), protein P4 (P4), protein P7 (P7), osmotin I (OSM; Singh et al. 1987a) and tobacco pathogenesis-related protein (PR-S; Cornelissen et al. 1986). (B): protein P2 (P2) and tobacco chitinase (CHI; Sinshi et al. 1987). (C): Protein L2 and the Rubisco small subunit of tobacco (RUBISCOS; Muller et al. 1983). (D): Protein L3 and the extrinsic membrane protein in the water-splitting complex of spinach (EM23kD; Vater et al. 1986). Homologous amino acid are boxed. The gaps that produce optional alignment are indicated by (-). Parentheses indicate that two amino acids were identified.

*Analysis of proteins in other cell types of tobacco*

The accumulation of proteins P1-P17 also was investigated in cultured tobacco cells grown in medium containing sucrose. It is clear from Fig. 4A that the presence of organic carbon sources in the medium had no effect on the accumulation pattern of proteins, but there were fewer Rubisco large and small subunits in photomixotrophic than in photoautotrophic cells. Analysis of

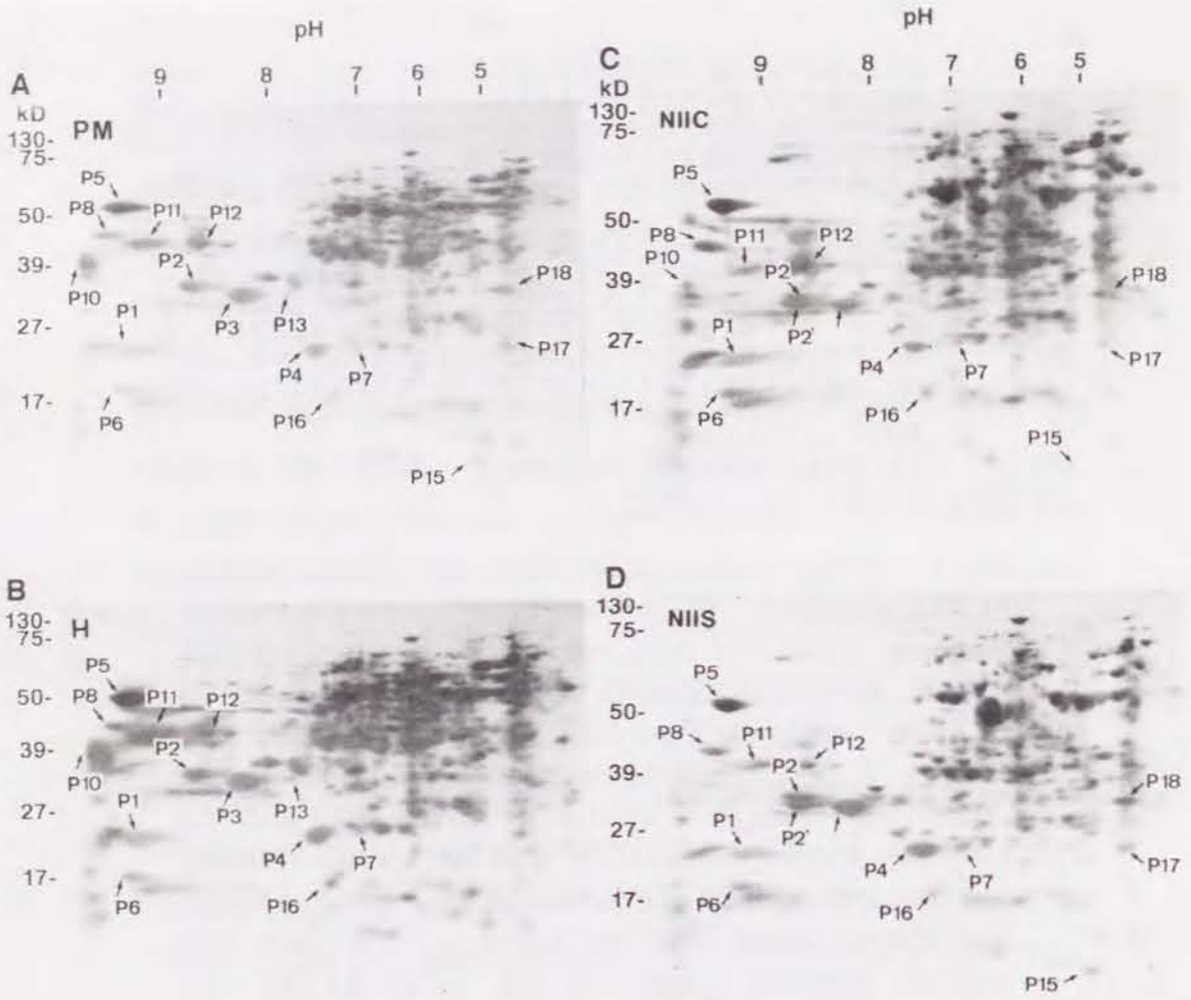



Fig. 4. 2D-PAGE of other cell types of tobacco. Proteins were extracted from the cells 1 week after inoculation or tissues by the method described in Materials and Methods. A: Photomixotrophic cultured cells (PM) to tobacco (NI line). B: Heterotrophic cultured cells (H) of tobacco (NI line). C: Cultured cells (NIIC) of a newly established line of tobacco (NII line) grown in the light. D: Regenerating shoots (NIIS) of tobacco (NII line).



heterotrophically cultured cells grown in the dark showed that illumination had no effect on the protein accumulation pattern, but that the proteins differed somewhat according to the cultured cell type (Fig. 4B).

For this analyses, I used only cells of a selected line (NI) which grows photoautotrophically. To examine whether other cultured lines of tobacco cells accumulate stress proteins, I next analyzed newly established cultured cells of tobacco (*Nicotiana tabacum* cv. Samsun NN) (Fig. 4C), and found that they accumulated protein P1-P7. Furthermore, when regenerating adventitious shoots were analyzed, a similar accumulation of proteins was also observed (Fig. 4D).

Osmotin is induced by NaCl stress (King et al. 1986, 1988, Singh et al. 1987a,b), and chitinase is formed as a pathogenesis-related protein as well as being induced by the plant hormone ethylene (Broglie et al. 1986, Shinshi et al. 1987), although both polypeptides have been found in cultured cells (King et al. 1986, 1988, Shinshi et al. 1987, Singh et al. 1987a,b). These results clearly indicate that cultured cells accumulate these stress proteins in large amounts. These results also suggest that being starved of a carbon source, light and dedifferentiation conditions do not themselves produce a stress response. Antoniw et al. (1981) reported that cultured *Nicotiana tabacum* cv. Xanthi nc callus also accumulated pathogenesis-related proteins, which suggests that suspension culture itself is not the origin of stress. Further analyses of other tissues of tobacco plants, i.e. old leaves, roots and leaves infected with Tobacco Mosaic Virus indicated that these tissues also accumulated the same molecular form of osmotin (P1 and P4) and chitinase (P2) (Fig. 5B-D). It has been

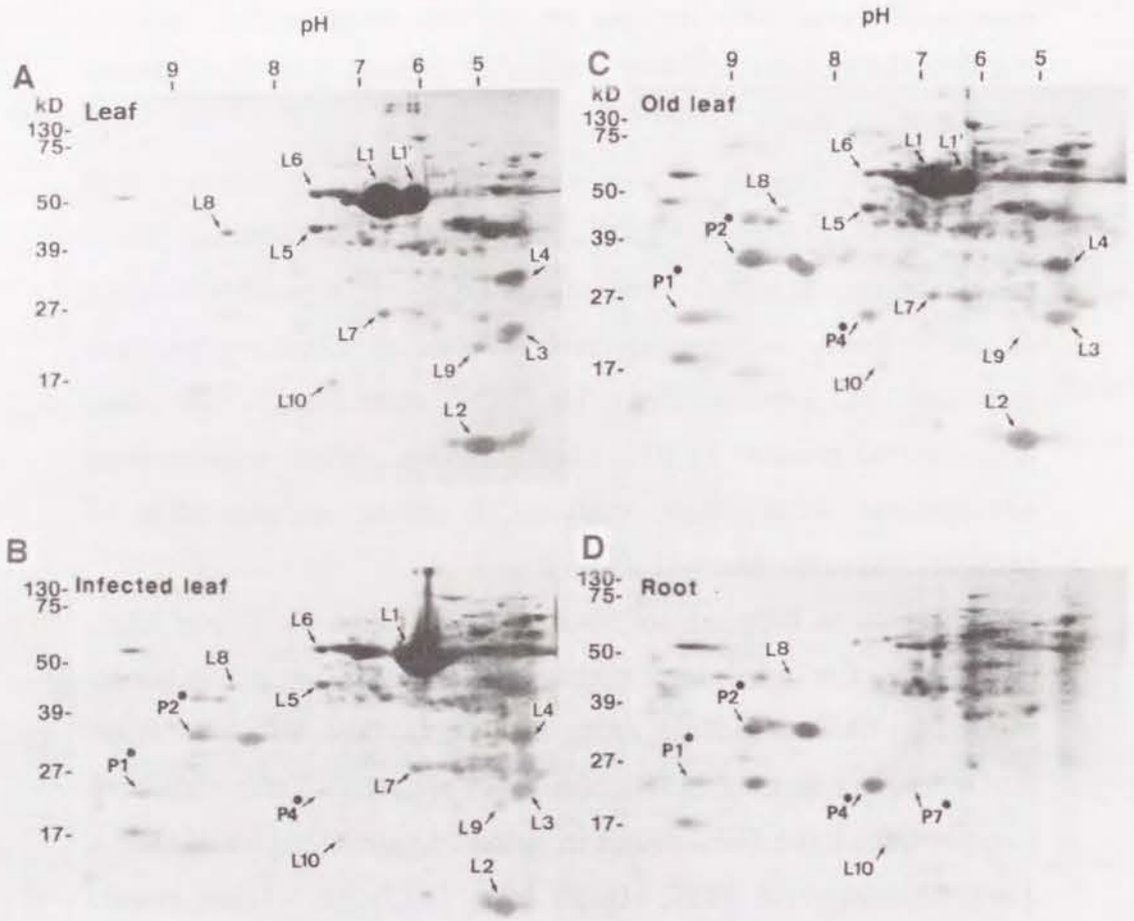



Fig. 5. 2D-PAGE of the tissues of tobacco plants. Proteins were extracted from the tissues by the method described in Materials and Methods. Same molecular form s of osmotin (P1*, P4* and P7*) or chitinase (P2*) as those in cultured green cells were identified. A: Half expanded healthy leaves (Leaf). B: Leaves infected with Tobacco Mosaic Virus (Infected leaf). C: Fully expanded old leaves (Old leaf). D: Roots of intact plants (Root).



reported that pathogen attack, elicitor treatment, and ethylene induce the same molecular forms of chitinase and β-1,3-glucanase in pea (Mauch et al. 1988). I will describe the regulatory mechanism on the gene expression of a stress protein, osmotin-like protein in cultured cells of tobacco more in detail in next chapter.

I also found several differences in the proteins that accumulated in this selected photoautotrophic cell line (NI) and in a newly established nonselected cell line (NII). My analysis of these proteins by microsequencing their $NH_2$-terminal amino acids has been hindered by the excessive amounts of stress proteins present. Organelles (e.g. the nucleus, vacuole and chloroplast) now must be isolated and studied to gain a clearer picture of and to identify the specific polypeptides that characterize this selected and nonselected lines.

CHAPTER IV

# MOLECULAR CLONING OF A cDNA FOR OSMOTIN-LIKE PROTEIN AND CHARACTERIZATION OF REGULATION OF THE GENE

## Section 1. Structure of a cDNA for osmotin-like protein from cultured tobacco cells.

In the previous chapter, I reported that P4 and P7, 26 kD proteins having $NH_2$-termial amino acid sequence homologous to osmotin, accumulate with osmotin to substantial levels in cultured tobacco (*Nicotiana tabacum* cv. Sumsun NN) cells. Osmotin and the analogous protein NP24 were first reported to accumulate in salt-adapted cells of tobacco and tomato (Singh et al. 1987, King et al. 1988). However, recent studies indicated that the expression of osmotin and osmotin-like proteins are regulated developmentally in intact plants with high levels of expression in roots (King et al. 1986, Neale et al. 1990, Singh et al. 1989, Fig. 5 in Chapter III). Furthermore, expression of these proteins was shown to be induced by Tobacco Mosaic Virus infection and wounding (Neale et al. 1990, Fig. 5 in Chapter III). Here, I report the nucleotide sequence of cDNA and its deduced amino acid sequence for an osmotin-like protein to learn more about the osmotin-like protein structure and its function in the stress response.



## MATERIALS AND METHODS

### *Cell Cultures*

A photomixotrophic culture (Nl line) of tobacco (*Nicotiana tabacum* cv. Samsun NN) was used as a material to isolate RNA. Photomixotrophic cells were maintained in modified Linsmaier-Skoog liquid medium with an addition of 3% sucrose on rotary or reciprocal shakers at 100 rpm and 25±2 ℃ in the light.

### *Construction of cDNA library, cloning of cDNA for osmotin-like protein and nucleotide sequence determination*

RNA was prepared from cultured tobacco cells by homogenization of cells in 6 M guanidine thiocyanate, followed by centrifugation over a cesium chloride cushion. Poly $(A)^+$ RNA was enriched by oligo (dT)-cellulose chromatography and then used as the template for the synthesis of cDNA transcripts. The cDNA transcripts formed were cloned in the plasmid pBluescript ($KS^+$) and used to transform *E.coli* HB101. The oligonucleotide mixtures for cDNA screening (Fig. 1) were designed from the $NH_2$-terminal amino acid sequence of the osmotin-like polypeptide, P4 (see Chapter III). Duplicate colony lifts on nitrocellulose filters were hybridized. The method of Sanger (1977) was used for the nucleotide sequence determination of the hybridization-positive clone pTOL1. The deduced amino acid sequence was compared with sequences in the NBRF protein identification resources using the IDEAS program.

```
          1   2   3   4   5   6   7
         Ser Gly Val Phe Glu Val His
         TCT GGT GTT TTT GAA GTT CAT
           C   C   C   C   G   C   C
           A   A   A               A
           G   G   G               G
         AGT
           C

Probe 1  AGG CCI CAI AAA CTT CAI GTA   21mer
                      G   C       G

          8   9   10  11  12  13  14  15  16  17  18  19  20  21
         Asn Asn Cys Pro Tyr Thr Val Trp Ala Ala Ala Thr Pro Val
         AAT AAT TGT CCT TAT ACT GTT TGG GCT GCT GCT ACT CCT GT
           C   C   C   C   C   C   C       C   C   C   C   C
                       A       A   A       A   A   A   A   A
                       G       G   G       G   G   G   G   G

Probe 2  TTA TTA ACA GGT ATA TGT CAA ACC CGI CGI CGI TGA GGT CA   41mer
           G   G   G       G   A   G
```

Fig. 1. The amino acid sequence and the corresponding nucleotide sequence of the synthetic inosine containing oligomer (Probe 1 and Probe 2) used to identify the cDNA clone for osmotin-like protein.

## RESULTS AND DISCUSSION

### *Isolation and characterization of cDNA clone*

A clone (pTOL1) was isolated from among about 10,000 cDNA clones. This clone contained a insert of length sufficient to encode a protein of 26 kD. Northern hybridization indicated that the message length was very similar to the length of the pTOL1 insert. These data suggest that pTOL1 contains an almost full-length cDNA of osmotin-like protein mRNA. Analysis of the complete nucleotide sequence of pTOL1 revealed that it contained one open reading frame encoding 251 amino acid residues followed by a termination codon (Fig. 2). The $NH_2$-terminal 38 amino acids of



```
      CT AAA AAG TCC ACT AAA ATG AGT CAC TTG ACA ACT TTT TTA GTG TTC   47
                              Met Ser His Leu Thr Thr Phe Leu Val Phe   10

 48  TTC CTC CTT GCC TTT GTG ACT TAT ACT TAT GCT TCC GGC GTA TTT GAA   95
 11  Phe Leu Leu Ala Phe Val Thr Tyr Thr Tyr Ala Ser Gly Val Phe Glu   26

 96  GTC CAT AAC AAT TGC CCA TAC ACC GTC TGG GCA GCG GCA ACC CCT GTA  143
 27  Val His Asn Asn Cys Pro Tyr Thr Val Trp Ala Ala Ala Thr Pro Val   42

144  GGC GGT GGC CGA CGT CTC GAA CGA GGT CAA AGT TGG TGG TTC TGG GCA  191
 43  Gly Gly Gly Arg Arg Leu Glu Arg Gly Gln Ser Trp Trp Phe Trp Ala   58

192  CCA CCT GGT ACT AAA ATG GCA CGT ATA TGG GGT CGT ACT AAT TGC AAC  239
 59  Pro Pro Gly Thr Lys Met Ala Arg Ile Trp Gly Arg Thr Asn Cys Asn   74

240  TTT GAT GGT GCT GGT AGA GGT TGG TGC CAA ACC GGT GAT TGT GGT GGA  287
 75  Phe Asp Gly Ala Gly Arg Gly Trp Cys Gln Thr Gly Asp Cys Gly Gly   90

288  GTC TTA GAA TGC AAA GGA TGG GGT AAA CCA CCA AAT ACC TTA GCC GAA  335
 91  Val Leu Glu Cys Lys Gly Trp Gly Lys Pro Pro Asn Thr Leu Ala Glu  106

336  TAC GCG TTG AAC CAA TTC AGC AAC TTA GAT TTC TGG GAC ATC TCT GTA  383
107  Tyr Ala Leu Asn Gln Phe Ser Asn Leu Asp Phe Trp Asp Ile Ser Val  122

384  ATT GAT GGA TTT AAC ATT CCT ATG TCT TTT GGC CCG ACT AAG CCT GGT  431
123  Ile Asp Gly Phe Asn Ile Pro Met Ser Phe Gly Pro Thr Lys Pro Gly  138

432  CCT GGA AAA TGT CAC GGA ATT CAA TGC ACA GCC AAC ATA AAT GGT GAA  479
139  Pro Gly Lys Cys His Gly Ile Gln Cys Thr Ala Asn Ile Asn Gly Glu  154

480  TGC CCT GGT TCA CTT AGG GTA CCT GGA GGA TGT AAC AAC CCA TGT ACA  527
155  Cys Pro Gly Ser Leu Arg Val Pro Gly Gly Cys Asn Asn Pro Cys Thr  170

528  ACA TTT GGA GGA CAA CAA TAT TGT TGC ACA CAA GGA CCA TGT GGT CCT  575
171  Thr Phe Gly Gly Gln Gln Tyr Cys Cys Thr Gln Gly Pro Cys Gly Pro  186

576  ACT GAG TTA TCA AGA TGG TTC AAA CAA AGA TGT CCT GAC GCC TAT AGT  623
187  Thr Glu Leu Ser Arg Trp Phe Lys Gln Arg Cys Pro Asp Ala Tyr Ser  202

624  TAT CCT CAA GAT GAT CCA ACA AGT ACA TTT ACT TGC ACA AGT TGG ACT  671
203  Tyr Pro Gln Asp Asp Pro Thr Ser Thr Phe Thr Cys Thr Ser Trp Thr  218

672  ACA GAT TAT AAG GTT ATG TTC TGT CCT TAT GGA AGT GCT CAC AAT GAA  719
219  Thr Asp Tyr Lys Val Met Phe Cys Pro Tyr Gly Ser Ala His Asn Glu  234

720  ACA ACA AAT TTC CCA TTG GAG ATG CCT ACA AGT ACT CAT GAA GTG GCC  767
235  Thr Thr Asn Phe Pro Leu Glu Met Pro Thr Ser Thr His Glu Val Ala  250

768  AAG TAG ATT CAT GAG TGA GTT AGC CAA CAC TTT CTT TGT TTA GTT TGC  815
251  Lys

816  TTG AAG ATG GTC ATT GTC TTT AAT TTG TCT TAG TTA CAC GCA TTT CTT  863

864  GTA ATT TGT TTT CTT GAA GTG GCT ATG ATC TGA ATA ACG ATC GAT CGA  911

912  TCA CAT ATA TTG TAC TTT TTC TTA CCC ATG ATG TTT TCA TTC TTC AAT  959

960  CAA GAG TGC ATT TGG ATT ATC GTG TAA AAA AAA AAA AAA AAA AAA
```

Fig. 2. Nucleotide and deduced amino acid sequence of osmotin-like protein. Underlined residues correspond to residues determined by sequential Edman degradation analysis. The processing site to yield mature osmotin-like protein is marked by an arrow. Double underlining indicates putative PEST sequences. Asterisks indicate a putative glycosylation site.

osmotin-like protein (P4), as determined by sequential Edman degradation analyses, correspond to the 22nd to 60th amino acid sequence deduced from the cDNA sequence and the calculated pI of the deduced amino acid sequence is 7.9, which is almost equal to the pI of P4 (7.5, see Table 1 in Chapter III). These correspondence confirmed that pTOL1 encodes osmotin-like protein rather than osmotin or another similar protein. Furthermore, comparison of the determined and deduced sequences suggests that osmotin-like protein is synthesized as a 29 kD precursor and processed to the 26 kD mature polypeptide. Hydrophobicity analysis using the DNASIS program (Hitachi Software Co. ) clearly shows the hydrophobic bias of the amino acids in the leader sequence (Fig. 3).

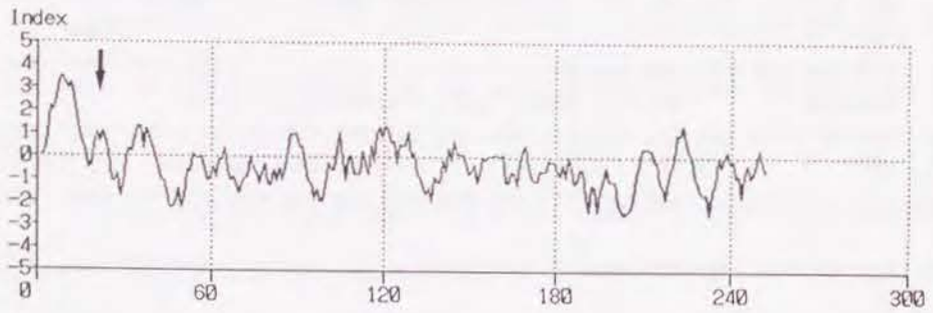



Fig. 3. Hydrophobicity analysis of osmotin-like protein. Hydrophobicity of osmotin-like protein was determined by a DNASIS program using the deduced amino acid sequence. The arrow indicates the processing site of the precursor to yield the mature protein.



*Sequence comparison of osmotin-like protein to related proteins*

When the amino acid sequence of osmotin-like protein was compared to sequences of other proteins, osmotin-like protein was found to be quite similar to osmotin (Singh et al. 1989), salt-induced tomato protein, NP24 (King et al. 1988), the thaumatin-like pathogenesis related protein of tobacco, PR-S (Cornelissen et al. 1986), maize α-amylase/trypsin inhibitor (Richardson et al. 1987) and thaumatin (Edens et al. 1982); (76%, 77%, 60%, 54%, 47% identical, respectively, Fig. 4). Furthermore, all of them are synthesized as pre-mature polypeptide, which possessed highly hydrophobic signal peptides, and processed to mature form. The sixteen cysteine residues involved in the disulfide bridges of thaumatin are perfectly conserved among the five proteins (Fig. 4). These results indicated that these polypeptides genes were originated from common ancestor gene.

Sequence analysis also indicated several characteristics of amino acid sequence unique to osmotin-like protein; The serine on the carboxyl side of the cleavage of the leader sequence of osmotin-like protein is an alanine in the other proteins (Fig. 4). Furthermore, osmotin-like protein possessed a putative glycosylation site (Fig. 2). Another interesting difference was that osmotin-like protein has the proline-glutamate-serine-threonine rich (PEST) sequence (Rogers et al. 1986) which is a characteristic sequence found in many rapidly turning over proteins (Fig. 2). These findings suggest that osmotin-like protein may have a function different from other proteins with similar sequences.


Fig. 4. Comparison of osmotin-like protein to other proteins. OLP: osmotin-like protein, OSM: osmotin (Singh et al. 1989), NP24: a 24kD salt-induced protein from tomato (King et al. 1988), THA: thaumatin II (Edens et al. 1982), PR-S: tobacco pathogenesis related protein (Cornelissen et al. 1986), MAI: maize α-amylase/trypsin inhibitor (Richardson et al. 1987). Asterisks indicate completely conserved cysteines. The completely conserved amino acids among osmotin-like protein and homologous proteins are boxed. The site of processing is shown by the arrow.



Osmotin highly accumulated in salt-adapted cells (Singh et al. 1987a,b) and its accumulation was induced by abscisic acid (Singh et al. 1987b, 1989). However, osmotin-like protein accumulated in less amounts in salt adapted cells (Sato et al. unpublished data) and the expression was not induced by the addition of abscisic acid (see next section). Furthermore, these results that the expression of osmotin-like protein gene was controlled by a different hormone ethylene support that osmotin-like protein has different function(s) from osmotin.

Sequence comparison revealed that osmotin-like protein was highly homologous to one of the anti-virus proteins recently isolated from the variety of tobacco (Fig. 5, Edelbaum et al. 1990). High homology suggested that osmotin-like protein might be a precursor of anti-viral protein gp22. Existence of PEST sequence would allow the rapid processing and activation of protein upon the infection of virus. Isolated cDNA of osmotin-like protein provide an opportunity to examine whether osmotin-like protein has anti-viral activity or not.

```
                                22
Osmotin like protein   SGVFEVHNNCPYTVWAAATPVGGGRRLERGQS
gp22 Fraction 24       :::::::::A:::::::::::::K::::S::

                               130
Osmotin like protein   MSFGPTKPGPGKCHGIQC
gp22 Fraction 20       ::::::::::::Y:V::A
```

Fig. 5. Sequence comparison of osmotin-like protein and CNBr-generated fragments of the anti-viral protein gp22 (Edelbaum et al. 1990). Numbers above the sequence indicate the position in osmotin-like protein. Colons indicate that the two proteins contain the same amino acid at that position.

Section 2. Regulation of the gene expression of osmotin-like protein by ethylene.

In Chapter III, I reported chitinase and β-1,3-glucanase, the most investigated pathogenesis related proteins, are also concomitantly found in cells when osmotin and osmotin-like proteins accumulate. Chitinase and β-1,3-glucanase are known to accumulate in response to the stress hormone, ethylene (Boller et al. 1983, Broglie et al. 1986, Felix and Meins 1987, Mauch et al. 1984). However, the expression of this enzyme is also influenced by auxin and kinetin, gibberelic acid and abscisic acid (Mohnen et al. 1985, Shinshi et al. 1987). Singh et al. (1989) reported that abscisic acid stimulates the synthesis of osmotin in cultured tobacco cells. I reported the primary structure of osmotin-like protein was quite similar to osmotin (76% identical in amino acid sequence). To learn more about the regulatory mechanisms of gene expression of osmotin and osmotin-like protein (OLP) and to clarify their functions in the stress response, I did the northern blot analysis and showed the influence of ethylene and ABA on the expression of osmotin-like protein.

## MATERIALS AND METHODS

### *Plant and Cell materials*

A photomixotrophic culture (NI line) of tobacco was used as a material to isolate RNA as described in the previous section. Tobacco plantlets grown under sterile conditions were used about 2 months after germination.



### *Northern blot analysis*

RNA was extracted from cultured cells at different growth phases or from cells treated with ethylene or ethylene-biosynthesis inhibitor as described below. Total RNA (10 μg) was separated on formaldehyde agarose gel and transferred to nylon membrane (Gene Screen Plus). $^{32}$P-labeled probe was prepared from cDNA insert of pTOL1 using the Random Primed DNA labeling Kit (BRL). Pre-hybridization was done at 42°C in 50% formamide, 0.65 M NaCl, 0.1 M Pipes-NaOH (pH 6.8), 5x Denhardt's solution, 0.5% SDS, 5 mM EDTA, 10% dextran sulfate and 100 μg/ml salmon testis DNA. Hybridization was carried out in the same solution with $^{32}$P-labeled probe at 42°C over night. Membranes were washed according to the manufacturer's instructions (Du Pont) and were autoradiographed. Under these hybridization conditions, cross-hybridization of OLP probe with osmotin-mRNA amplified by PCR was negligible (data not shown).

### *Ethylene treatment and inhibition of ethylene biosynthesis in cultured tobacco cells and plantlets*

To examine the effect of ethylene on the expression of the gene for osmotin-like protein, the following experiments were conducted: The endogenous ethylene biosynthesis of cultured cells was suppressed by the cultivation of cells in medium containing 0.25 mM $Co(NO_3)_2$ for 5 days, and the induction of expression of osmotin-like protein mRNA by ethylene was examined after the injection of ethylene (final concentration 100 μl/l) into the culture flask through the silicone cap with an air-tight microsyringe. After certain time periods, cells were harvested, frozen in liquid nitrogen and stored at -80°C for extraction of RNA. The induction

of expression of osmotin-like protein was also examined in intact plantlets; plantlets which were grown aseptically were treated with ethephon (final concentration 50 μg/ml), an ethylene generator. After one week of incubation, RNA was extracted.

The effect of abscisic acid was also examined with the cells in which ethylene biosynthesis had been suppressed for 5 days. ABA (final concentration 10 μM) was added to the cultured medium as a sterile solution. RNA was prepared as described above.

## RESULTS AND DISCUSSION

The expression of osmotin-like protein was first examined with cultured cells at different growth phases. Messenger RNA isolated from cells at lag phase (day 3), exponential phase (day 7), and stationary phase (day 14) was separated by denaturing gel electrophoresis, blotted onto a filter, and probed with pTOL1. The autoradiogram clearly shows that older cells contained elevated levels of OLP message (Fig. 1).

Fig. 1 also indicate that the $Co(NO_3)_2$, an inhibitor of ethylene biosynthesis, considerably inhibited the increase of OLP transcripts in cultured cells, even cells in stationary phase. The effect of ethylene was further examined with cells in which ethylene biosynthesis was suppressed by $Co(NO_3)_2$. Fig. 2 clearly indicated that inhibitory effect of $Co(NO_3)_2$ on the gene expression of osmotin-like protein was compensated by the addition of ethylene. That is, the addition of ethylene to the cells in which ethylene biosynthesis was suppressed by $Co(NO_3)_2$ increased the OLP gene transcript on one day after treatment while induction of OLP gene transcription was observed in cells without $Co(NO_3)_2$



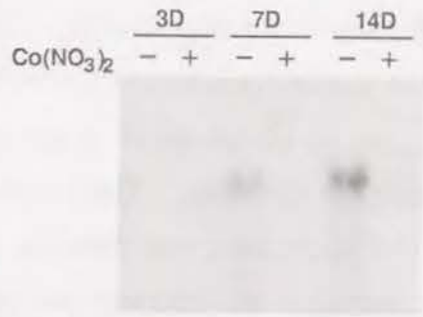



Fig. 1. Northern blot analyses of RNA from photomixotrophic cells. The effect of the ethylene synthesis inhibitor $Co(NO_3)_2$ on the expression of osmotin-like protein mRNA was examined using cultured cells at different growth phase (3, 7 and 14 days after inoculation). The + and - above the lanes indicate that RNA was prepared from cells treated with or without $Co(NO_3)_2$, respectively. Total RNA (10 μg) was separated by agarose gel electrophoresis and transferred to nylon membrane. Osmotin-like protein mRNA was detected using labeled pTOL1 insert.

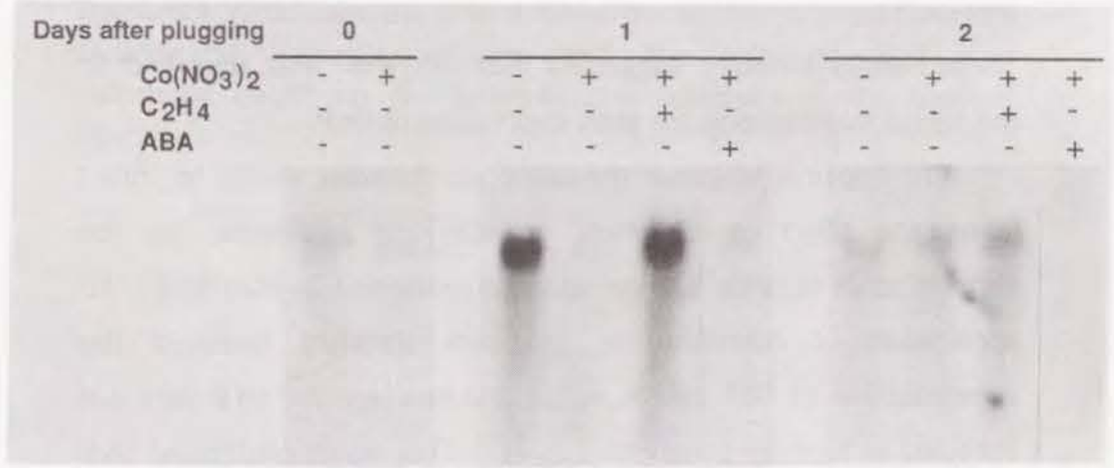



Fig. 2. Effect of ethylene and ABA on the expression of osmotin-like protein gene. After 5 days of culture with (+) or without (-) $Co(NO_3)_2$, culture flask was plugged, and ethylene or ABA was injected into the culture flask. RNA was isolated immediately, or after 1 or 2 days and separated and detected as described for Fig.1.

treatment after plugging of flask. It is supposed that high accumulation of ethylene produced by the cells in the plugged flask induced the expression (data not shown). However, ABA could not compensate the inhibitory effect of $Co(NO_3)_2$ on the OLP expression, while Singh et al. (Singh et al. 1989) reported that ABA induces the message for osmotin. These results suggested that the regulation of OLP expression was different from that of osmotin. But recently, LaRosa et al. reported that transcription is also induced by ethylene. It is interesting to examine the effect of $Co(NO_3)_2$ on the induction of osmotin transcription.

The time course of induction of OLP transcription by ethylene indicated that the OLP mRNA level began to increase after 4 hours of injection of ethylene and reached the maximum (about 17 times of initial level) after 16 hours, and decreased to the initial level after 48 hours (Fig. 3). On the other hand, the OLP mRNA level in the cells without ethylene treatment was fairly constant. These results strongly suggested that ethylene was involved in the signal transduction for gene expression of OLP.

To confirm whether the same mechanism works in intact plant, the effect of ethephon, an ethylene generator, on the expression of osmotin-like protein was examined in plantlets. An application of ethephon on plantlets strongly induced the accumulation of OLP mRNA, while the message for OLP was not detected in healthy plantlets (Fig. 4). This result confirmed that gene expression of osmotin-like protein was regulated by the ethylene as well as those of chitinase and β-1,3-glucanase (Boller et al. 1983, Broglie et al. 1986, Felix and Meins 1987, Mauch et al. 1984).



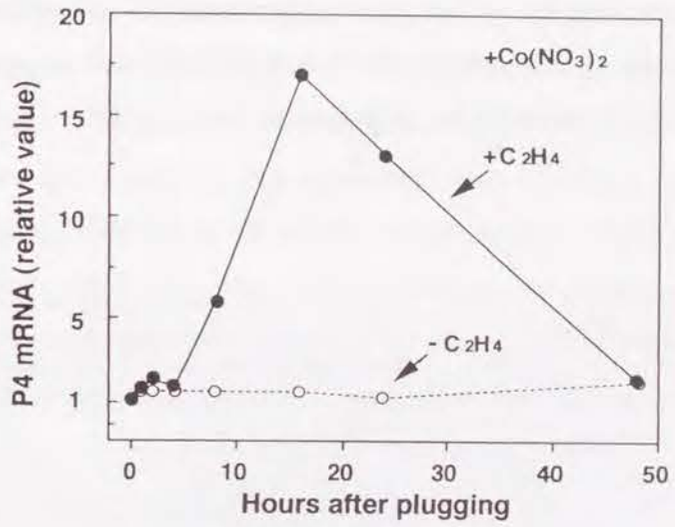



Fig. 3. Induction of mRNA for osmotin-like protein by ethylene. After 5 days of culture with $Co(NO_3)_2$ , cultures flasks were plugged and ethylene was injected into the culture flask (●) or not (○). RNA was isolated immediately and 1, 2, 4, 8, 16, 24, 48 hours later, and separated and detected as described for Fig. 1. Hybridization intensity was determined by the AMBIS radioanalytic imaging system and the relative value was calculated based on the hybridization intensity at the time of injection.


Fig. 4. Northern blot analysis of RNA from young leaves. RNA was prepared from the plantlets treated with (+) or without (-) the ethylene generator ethephon. RNA was separated and detected as described for Fig. 1.

The effect of ethylene was also examined for the expression of other genes (Fig. 5). The accumulation of mRNA of chitinase was suppressed by the addition of $Co(NO_3)_2$ and this suppression was overcome by the addition ethylene as that of OLP. On the other hand, the level of mRNA for *cab* gene encoding light harvesting Chl *a*/*b*-binding protein, which is known to accumulate in response to the light, was not affected by the addition of $Co(NO_3)_2$. The effect of ethylene was not examined for *cab* , because the plugging induced the rapid decrease. In all genes examined, the mRNA level decreased after 2 days of plugging. This indicated that anaerobic condition produced by plugging would inhibit the transcription of those genes and/or increase their degradation. Above results also mean that the effect of $Co(NO_3)_2$ was more specific to the genes in which expression ethylene is involved.

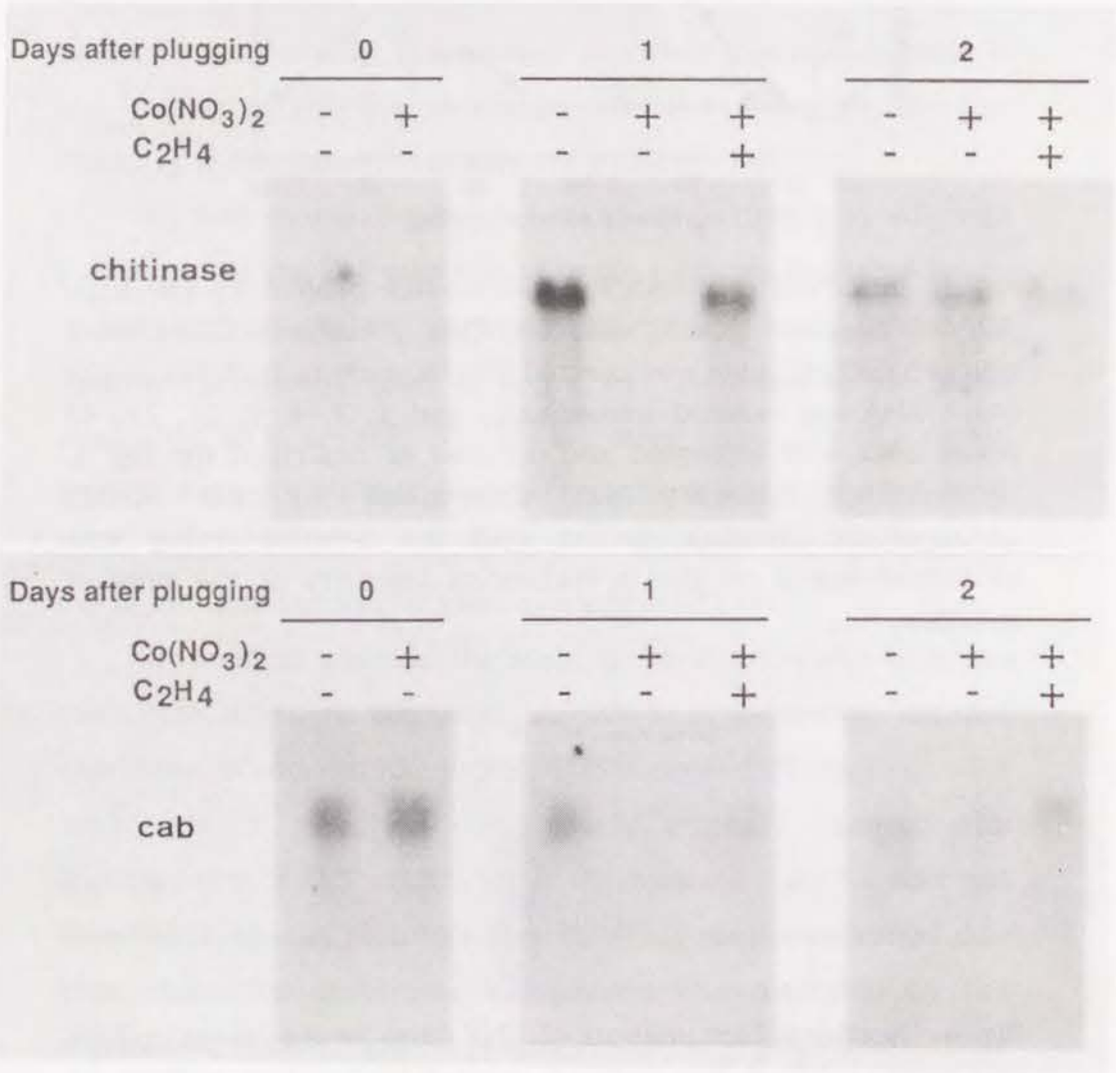



Fig. 5. Effect of ethylene on the expression of chitinase and cab gene. RNA was prepared and separated as described for Fig. 1, and probed with cDNA for chitinase and cab.



These data indicated that ethylene is a potent regulator of gene expression of osmotin-like protein. The nature of ethylene which stimulates the senescence of chloroplasts (Choe and Whang 1986) well explains why the chloroplasts of cultured cells were less developed than those in green leaves (see Chapter I). Cultured cells provide a good material to investigate the regulation of gene expression by ethylene and its signal transduction.

## CONCLUSIONS

I have investigated regulation of gene expression in cultured green cells of *Nicotiana tabacum*. My findings reported in the preceding chapters are summarized as follows:

### CHAPTER I

Ultrastructural studies on the chloroplasts in photoautotrophically and photomixotrophically cultured cells of tobacco (*Nicotiana tabacum* cv. Samsun NN) indicated that cultured green cells are useful materials to study the biogenesis of chloroplasts, especially the division of chloroplasts. Analyses of the photosynthetic characteristics of photoautotrophically cultured cells as well as those of photomixotrophically cultured cells and green leaves revealed that cultured tobacco cells had lower chlorophyll contents than cells of green leaves on a fresh weight basis. The chlorophyll content per chloroplast was almost the same in both types of cell, and the chloroplast number per cell accounted for only small differences in the cellular chlorophyll content. This indicated that the larger cell volume of cultured cells is the main factor in the difference in the chlorophyll content of these cells. Photosynthetic activity measurements also showed differences in the chloroplasts of cultured and leaf cells. The maximum activities of photosystem I and the Hill reaction for the cultured cells were about half those for leaf cells on a per unit chlorophyll basis. Moreover, photoautotrophic cells had relatively constant photosystem I and Hill reaction activities during growth; whereas, these activities in leaf cells reflected developmental changes in the chlorophyll content on a fresh weight basis. Lithium dodecyl sulfate-polyacrylamide gel electrophoresis



showed qualitatively similar thylakoid polypeptide compositions for cultured and leaf cells at all stages of growth even though there were quantitative decreases in the contents of several polypeptides in the cultured green cells (especially in photomixotrophic cells) in comparison to the polypeptide contents of tobacco leaves. I speculate that the lower photosynthetic activity of the cultured cells may be caused by this reduction in the contents of certain thylakoid polypeptides.

### CHAPTER II

The effects of herbicides with different primary modes of action were examined on the growth of photoautotrophic, photomixotrophic, and heterotrophic cultures of tobacco cells. These responses were compared with those of tobacco seedlings to the same herbicides. Herbicides, which primarily inhibit or disturb photosynthetic processes, suppressed the growth of photoautotrophic cells most strongly, as compared to photomixotrophic and heterotrophic cells (atrazine, DCMU, paraquat). Herbicides having a primary mode of action other than the inhibition of photosynthetic processes, suppressed the growth of all types pf cultured cells as similar concentrations (2,4-D, diphinamid, glyphosate, DNBP, sodium chlorate, bialaphos, DTP), but the photoautotrophic cells were still the most sensitive to all kinds of herbicides except sodium chlorate. Furthermore, photoautotrophic cells responded to most of the herbicides as did the seedlings, with the exception of glyphosate and diphinamid. Thus, photoautotrophically cultured cells are considered as a model system to study the effects of herbicides.

CHAPTER III

Characterization and microsequencing of the polypeptides that accumulated in large amounts in photoautotrophically cultured cells of tobacco revealed four $NH_2$-terminal amino acid sequences that were highly homologous to those of the known stress proteins, osmotin and chitinase. Further analyses of the tobacco cell line grown with sucrose in light and in darkness, as well as analyses of newly established cultured cells and regeneration adventitious shoots, clearly showed the all the *in vitro* cultured cells accumulated these stress proteins. The accumulation of these proteins were also observed in old leaves, roots, and leaves infected with Tobacco Mosaic Virus, but not in young healthy leaves.

CHAPTER IV

The primary structure of an osmotin-like protein was deduced from the nucleotide sequence of a cDNA clone made from mRNA isolated from cultured tobacco cells. The amino acid sequence indicated that osmotin-like protein is synthesized as 29kD polypeptide with a hydrophobic leader peptide of 21 amino acids, and then processed to a 26kD polypeptide. The sequence analysis also suggested that osmotin-like protein possessed a putative glycosylation site as well as PEST sequence, neither of which were found in osmotin or tobacco pathogenesis-related protein (PR-S), although the amino acid sequence of osmotin-like protein is 76 % and 60 % identical to the sequences of these other proteins, respectively. Osmotin-like protein mRNA was found to accumulate to high levels when cells approached stationary stage. The expression of osmotin-like protein was suppressed when cells



were treated with the ethylene biosynthesis-inhibitor, $Co(NO_3)_2$, but this suppression was overcome by the injection of ethylene. Another plant hormone, abscisic acid had no effect on the expression of osmotin-like protein. These results and the previous findings indicated that ethylene is a potent regulator of gene expression in cultured cells.

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REFERENCES

Amrhein, N., J. Schab and H. C. Steinrucken. (1980). Naturwissenchaften. 67: 356-358.

Antoniw, J. F., J. S. H. Kush, D. G. A. Walkey and R. F. White. (1981). "The presence of pathogenesis-related proteins in callus of Xanthi-nc tobacco." Phytopath. Z. 101: 179-184.

Ashton, F. M. and A. S. Crafts. (1981). Mode of action of herbicides. New York, John Wiley and Sons.

Bauw, G., M. De Loose, D. Inze, M. Van Montagu and J. Vandekerckhove. (1987). "Alternations in the phenotype of plant cells studied by $NH_2$-terminal amino acid-sequence analysis of proteins electroblotted from two-dimensional gel separated total extracts." Proc. Natl. Acad. Sci. USA. 84: 4806-4810.

Bergman, L. (1967). "Wachstum grüner Suspensionskulturen von *Nicotiana tabacum* with var."Samsun" mit $CO_2$ als Kohlenstoffquelle." Planta. 74: 243-249.

Blair, L. C., C. J. Chastain and J. M. Widholm. (1988). "Initiation and characterization of a cotton (*Gossypium hirsutum* L.) photoautotrophic cell suspension culture." Plant Cell Reports. 7: 266-269.

Boller, T., A. Gehri, F. Mauch and U. Voegeli. (1983). "Chitinase in bean leaves: Induction by ethylene, purification, properties, and possible function." Planta. 157: 22-31.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the protein-dye binding." Anal. Biochem. 72: 248-254.

Brangeon, J. and A. Nato. (1981). "Heterotrophic tobacco cell cultures during greening I.Chloroplast and cell development." PHYSIOL. PLANT. 53: 327-334.

Broglie, K. E., J. J. Gaynor and R. M. Broglie. (1986). "Ethylene-regulated gene-expression: molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*." Proc. Natl. Acad. Sci. USA. 83: 6820-6824.

Camp, P. J., S. C. Huber, J. J. Burke and D. E. Moreland. (1982). "Biochemical changes that occur during senescence of wheat leaves: Bias for the reduction of photosynthesis." Plant Physiol. 70: 1641-1646.

Carrier, P., P. Chagvardieff and P. Tapie. (1989). "Comparison of the oxygen exchange between photosynthetic cell suspensions and detached leaves of *Euphorbia characias* L." Plant Physiol. 91(3): 1075-1079.

Chaleff, R. S. and T. B. Ray. (1984). Science. 223: 1148-1151.

Choe, H. T. and M. Whang. (1986). "Effects of ethephon on aging and photosynthetic activity in isolated chloroplasts." Plant Physiol. 80: 305-309.

Chua, N. H., K. Mutlin and P. Bennoun. (1975). "A chlorophyll protein complex lacking in photosystem I mutants of Chlamydomonas reinhardtii." J. Cell. Biol. 67: 361-377.

Cornelissen, B. J. C., R. A. M. Hooft van Huijsduijnen and J. F. Bol. (1986). "A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin." Nature. 321: 531-532.

Cséplö, A. and P. Medgyesy. (1986). "Characteristic symptoms of photosynthesis inhibition by herbicides are expressed in photomixotrophic tissue cultures of *Nicotiana*." Planta. 168: 24-28.

Dalton, C. C. (1980). "Photoautotrophy of spinach cells in continuous culture: Photosynthetic development and sustained photoautotrophic growth." J. Exp.Bot. 31(122): 791-804.

Davis, D. G. and R. H. Shimabukuro. (1980). "Studies of herbicide toxicity and mode of action using isolated mesophyll cells and callus derived suspensions." Can. J. Bot. 58: 1482-1489.

Delepelaire, P. and N. H. Chua. (1979). "Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of thylakoid membranes at 4 °C: Characterizations of two additional chlorophyll a-protein complexes." Proc. Natl. Acad. Sci. USA. 76: 111-115.



Dodge, A. D. (1983). The mode of action of herbicides. Progress in Pesticide Biochemistry and Toxicology. New York, John Wiley & Sons Ltd.

Edelbaum, O., N. Ilan, G. Grafi, N. Sher, Y. Stram, D. Novick, N. Tal, I. Sela and M. Rubinstein. (1990). "Two antiviral proteins from tobacco: Purification and characterization by monoclonal antibodies to human ß-interferon." Proc. Natl. Acad. Sci. USA. 87: 588-592.

Edens, L., L. Heslinga, R. Klok, A. M. Ledeboer, J. Maat, M. Y. Toonen, C. Visser and C. T. Verrips. (1982). "Cloning of cDNA encoding the sweet-tasting plant protein thaumatin and its expression in *Escherichia coli*." Gene. 18: 1-12.

Ellis, J. and R. M. Leech. (1985). "Cell size and chloroplast size in relation to chloroplast replication in light-grown wheat leaves." Planta. 165: 120-125.

Epel, J. R. and J. Neumann. (1973). "The mechanism of the oxidation of ascorbate and $Mn^{2+}$ by chloroplasts. The role of the radical superoxide." Biochem. Biophys. Acta. 325: 520-529.

Felix, G. and F. Meins. (1987). "Ethylene regulation of β-1,3 glucanase in tobacco." Planta. 172: 386-392.

Ford, D. M. and R. Shibles. (1988). "Photosythesis and other traits in relation to chloroplast number during soybean leaf senescence." Plant Physiol. 86: 108-111.

Fosket, D. E. (1968). "Cell division and the differentiation of wound-vessel members in cultured stem segments of *Coleus*." Proc. Natl. Acad. Sci. USA. 59: 1089-1096.

Gillot, M. A., G. Erdös and D. E. Buetow. (1991). "Light-induced chloroplast differentiation in soybean cells in suspension culture." Plant Physiol. 96: 962-970.

Goldstein, C. S. and J. M. Widholm. (1990). "Photosynthetic characterization of photoautotrophic cells cultured in a minimal medium." Plant Physiol. 94: 1641-1646.

Gressel, J., S. Zilkah and G. Ezra. (1978). Herbicide action, resistance and screening in cultures vs. plants. Frontiers of plant tissue culture. Calgary, University of Calgary.

Harak, A. and R. D. Hill. (1972). "Adenosine triphosphatase of bean plastids: its properties and site of formation." Plant Physiol. 49: 365-370.

Herzbeck, H. and W. Hüsemann. (1985). Photosynthetic carbon metabolism in photoautotrophic cell suspension cultures of *Chenopodium rubrum* L. Primary and secondary metabolism of plant cell cultures. Berlin, Heidelberg, New York, Tokyo, Springer.

Horn, M. E., J. H. Sherrard and J. M. Widholm. (1983). "Photoautotrophic growth of soybean cells in suspension culture. I. Establishment of photoautotrophic cultures." Plant Physiol. 72: 426-429.

Horn, M. E. and J. M. Widholm. (1984). Aspects of photosynthetic plant tissue cultures. Applications of Genetic Engineering to Crop Improvement. Boston, Martinus Nijhof Dr W.Junk.

Hüsemann, W., A. Plohr and W. Barz. (1979). "Photosynthetic characteristics of photomixotrophic and photoautotrophic cell suspension cultures of *Chenopodium rubrum* ." Protoplasma. 100: 101-112.

Jensen, R. G. and J. A. Bassham. (1966). "Photosynthesis by isolated chloroplasts." Proc. Natl. Acad. Sci. USA. 56: 1095-1101.

Kameya, T. (1972). "Cell elongation and division of chloroplasts." J. Exp. Bot. 23: 62-64.

Kanehisa, M. (1982). "Los Alamos sequence analysis package for nucleic acids and proteins." Nucleic Acid Res. 10: 183-196.

Kawakubo, K., M. Shindo and T. Konotsune. (1979). "A mechanism of chlorosis caused by 1,3-dimethyl-4-2,4-dichlorobenzoyl-5-hydroxy pyrazole: A herbicidal compound." Plant Physiol. 64: 774-779.

King, G. J., C. E. Hussey Jr. and V. A. Turner. (1986). "A protein induce by NaCl in suspension cultures of *Nicotiana tabacum* accumulates in whole plant roots." Plant Mol. Biol. 7: 441-449.

King, G. J., V. A. Turner, C. E. Hussey Jr., E. S. Wurtele and S. M. Lee. (1988). "Isolation and characterization of a tomato cDNA clone which codes for a salt-induced protein." Plant Mol. Biol. 10: 401-412.



Kondo, Y., T. Shomura, Y. Ogawa, T. Tsuruoka, H. Watanabe, K. Totsukawa, T. Suzuki, C. Moriyama, J. Yoshida, S. Inouye and T. Nishida. (1973). Sci. reports of Meiji Seika Kaisya. 13: 34-41.

La Rosa, P. C., P. M. Hasegawa and R. A. Bressan. (1984). "Photoautotrophic potato cells: Transition from heterotrophic to autotrophic growth." Physiol. Plant. 61: 279-286.

LaRosa, P. C., Z. Chen, D. E. Nelson, N. K. Singh, P. M. Hasegawa and R. A. Bressan. (1992). "Osmotin gene expression is posttranscriptionally regulated." Plant Physiol. 100: 409-415.

Leason, M., D. Cunliffe, D. Parkin, P. J. Lea and B. J. Miflin. (1982). "Inhibition of pea (*Pisum sativum* ) glutamine synthetase (EC-6.3.1.2) by methionine sulfoximine phosphinothricin and other glutamate analogs." Phytochemistry. 21: 855-857.

Matsudaira, P. (1987). "Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes." J. Biol. Chem. 262: 10035-10038.

Mauch, F., L. A. Hadwiger and T. Boller. (1984). "Ethylene: Symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors." Plant Physiol. 75: 607-611.

Mauch, F., L. A. Hadwiger and T. Boller. (1988). "Antifungal hydrolases in Pea tissue. I. Purification and characterization of two chitinase and two β-1,3-glucanases differentially regulated during development in response to fungal infection." Plant Physiol. 87: 325-333.

Mohnen, D., H. Shinshi, G. Felix and F. Meins. (1985). "Hormonal regulation of β-1,3-glucanase messenger RNA levels in cultured tobacco tissues." EMBO J. 4: 1631-1635.

Muller, K. D., J. Salnikow and J. Vater. (1983). "Amino acid sequence of the small subunit of d-ribulose bisphosphate carboxylase/oxygenase from *Nicotiana tabacum*." Biochim. Biophys. Acta. 742: 78-83.

Mullet, J. E., J. J. Burke and C. J. Arntzen. (1980). "Chlorophyll proteins of photosystem I." Plant Physiol. 65: 814-822.

Nafziger, E. D., J. M. Widholm, H. C. Steinrucken and J. L. Killmer. (1984). "Selection and characterization of a carrot cell line tolerant to glyphosate." Plant Physiol. 76: 571-574.

Nato, A., S. Bazetoux and Y. Mathieu. (1977). "Photosynthetic capacities and growth characteristics of Nicotiana tabacum (cv. Xanthi) cell suspension cultures." Physiol. Plant. 41: 116-123.

Nato, A., Y. Mathieu and J. Brangeon. (1981). "Heterotrophic tobacco cell cultures during greening II, Physiological and biochemical aspects." Physiol. Plant. 53: 335-341.

Nato, A., J. Hoarau, J. Brangeon, B. Hirel and A. suzuki. (1985). Regulation of carbon and nitrogen assimilation pathways in tobacco cell suspension cultures in relation with ultrastructural and biochemical development of the photosynthetic apparatus. Primary and secondary metabolism of plant cell cultures. Berlin, Heidelberg, New York, Tokyo, Springer.

Neale, A. D., J. A. Wahleithner, M. Lund, H. T. Bonnett, A. Kelly, D. R. Meeks-Wagner, W. J. Peacock and E. S. Dennis. (1990). "Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation." Plant Cell. 2: 673-684.

Neumann, K. H. and L. Bender. (1987). Photosynthesis in cell and tissue culture systems. Plant Biology. New York, Alan R. Liss, Inc.,.

Nishida, K., F. Sato and Y. Yamada. (1980). "Photosynthetic carbon metabolism in photoautotrophically and photomixotrophically cultured tobacco cells." Plant Cell Physiol. 21(1): 47-55.

O'Farrell, P. H. (1975). "High resolution two-dimensional electrophoresis of proteins." J. Biol. Chem. 250: 4007-4021.

Payne, G., W. Middlesteadt, S. Williams, N. Desai, D. Parks, S. Dincher, M. Carnes and J. Ryals. (1988). "Isolation and nucleotide sequence of a novel cDNA clone encoding the major form of pathogenesis-related protein R." Plant Mol. Biol. 11: 223-224.

Peel, E. (1982). "Photoautotrophic growth of suspension cultures of *Asparagus offecinalis* L. Cells in turbidostats." Plant Science Letters. 24: 147-155.



Possingham, J. V. and w. Saurer. (1969). "Changes in chloroplast number per cell during leaf development in Spinach." Planta. 86: 186-194.

Rey, P., E. Eymery and G. Peltier. (1990). "Effects of $CO_2$-enrichment and of aminoacetonitorile on growth and photosynthesis of photoautotrophic calli of *Nicotiana plumbaginifolia*." Plant Physiol. 93: 549-554.

Richardson, M., S. Valdes-Rodriguez and A. Blanco-Labra. (1987). "A possible function of thaumatin and a TMV-induced protein suggested by homology to a maize inhibitor." Nature. 327: 432-434.

Roeske, C. A., J. M. Widholm and W. L. Ogren. (1989). "Photosynthetic carbon metabolism in photoautotrophic cell suspension cultures grown at low and high carbon dioxide." Plant Physiol. 91(4): 1512-1519.

Roger, S. M. D., W. L. Ogren and J. M. Widholm. (1987). "Photosynthetic characteristics of a photoautotrophic cell suspension culture of soybean." Plant physiol. 84: 1451-1456.

Rogers, S., R. Wells and M. Rechsteiner. (1986). "Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis." Science. 234: 364-368.

Sanger, F., S. Nicklen and A. R. Coulson. (1977). "DNA sequencing with chain terminating inhibitors." Proc. Natl. Acad. Sci. USA. 74: 5463-5467.

Sato, F., K. Asada and Y. Yamada. (1979). "Photoautotrophy and the photosynthetic potential of chlorophyllous cells in mixotrophic cultures." Plant Cell Physiol. 20: 193-200.

Sato, F., K. Nishida and Y. Yamada. (1980). "Activities of carboxylation enzymes and products of $^{14}CO_2$ fixation in photoautotrophically cultured cells." Plant Sci. Lett. 20: 91-97.

Sato, F., N. Koizumi and Y. Yamada. (1988). "Purification and characterization of phosphoenolpyrubate carboxylase of photomixotrophically cultured green tobacco cells." Plant Cell Physiol. 29(2): 329-337.

Sato, F., Y. Shigematsu and Y. Yamada. (1988). "Selection of atrazine-resistant tobacco cell line having a mutant *psb*A gene." Mol. Gen. Genet. 214: 358-361.

Sato, F., Y. Yamada, S. S. Kwak, K. Ichinose, M. Kishida, N. Takahashi and S. Yoshida. (1991). "Photoautotrophic cultured plant cells: A novel system to survey new photosynthetic electron transport inhibitors." Z. Naturforsch. 46c: 563-568.

Seeni, S. and A. Gnanam. (1982). "Growth of photoheterotrophic cells of peanut (*Arachis hypogaea* L.) in still nutrient medium." Plant Physiol. 70: 815-822.

Shinshi, H., D. Mohnen and F. Meins Jr. (1987). "Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin." Proc. Natl. Acad. Sci. USA. 84: 89-93.

Shuster, A. M. and E. Davies. (1983). "Ribonucleic acid and protein metabolism in pea epicotyls. I. The aging process." Plant Physiol. 73: 809-816.

Singer, S. and C. McDaniel. (1985). "Selection of glyphosate-tolerant tobacco calli and the expression of this tolerance in regenerated plants." Plant Physiol. 78: 411-416.

Singh, N. K., C. A. Bracker, P. M. Hasegawa, A. K. Handa, S. Buckel, M. A. Hermodson, E. Pfankoch, F. E. Regnier and R. Bressan. (1987). "Characterization of osmotin, a thaumatin-like protein associated with osmotic adaptation in plant cells." Plant Physiol. 85: 529-536.

Singh, N. K., P. C. La Rosa, A. K. Handa, P. M. Hasegawa and R. A. Bressan. (1987). "Hormonal regulation of protein synthesis associated with salt tolerance in plant cells." Proc. Natl. Acd. Sci. USA. 84: 739-743.

Singh, N. K., D. E. Nelson, D. Kuhn, P. M. Hasegawa and R. A. Bressan. (1989). "Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential." Plant Physiol. 90: 1096-1101.

Thayer, S. S. and O. Björkman. (1990). "Leaf xanthophyll content and composition in sun and shade determined by HPLC." Photosynthesis Research. 23: 331-343.



Toyama, S. (1980). "Electron microscope studies on the morphogenesis of plastids. X. Ultrastructural changes of chloroplasts in morning glory leaves exposed to ethylene." Amer. J. Bot. 67(5): 625-635.

Tsuji, H., K. Naito, I. Hatakeyama and K. Ueda. (1979). "Benzyladenine-induced increase in DNA content per cell, chloroplast size, and chloroplast number per cell in intact bean leaves." J. Exp. Bot. 30: 1145-1151.

Tsuzuki, M., S. Miyachi, F. Sato and Y. Yamada. (1981). "Photosynthetic characteristics and carbonic anhydrase activity in cells cultured photoautotrophically and mixotrophically and cells isolated from leaves." Plant Cell Physiol. 22(1): 51-57.

Tyler, R. T., W. G. W. Kurz and B. D. Panchuk. (1986). "Photoautotrophic cell suspension cultures of periwinkle (*Catharanthus roseus* (L.) G.Don): Transition from heterotrophic to photoautotrophic growth." Plant Cell Reports. 3: 195-198.

van Kan, J. A. L., M. D. van de Rhee, D. Zuidema, B. J. C. Cornelissen and J. F. Bol. (1989). "Structure of tobacco genes encoding thaumatin-like proteins." Plant Mol. Biol. 12: 153-155.

Vater, J., J. Salnikow and C. Jansson. (1986). "N-terminal sequence determination and secondary structure analysis of extrinsic membrane proteins in the water-splitting complex of spinach." FEBS Lett. 203: 230-233.

Widholm, J. M. (1992). "Properties and uses of photoautotrophic plant cell cultures." International Review of Cytology. 132: 109-129.

Xu, C., L. C. Blair, S. M. D. Rogers, Govindjee and J. M. Widholm. (1988). "Characteristics of five new photoautotrophic suspension cultures including two *Amaranthus* species and a cotton strain growing on ambient $CO_2$ levels." Plant Physiol. 88: 1297-1302.

Yamada, Y. and F. Sato. (1978). "The photoautotrophic culture of chlorophyllous cells." Plant Cell Physiol. 19: 691-699.

Yamada, Y., F. Sato and K. Watanabe. (1982). Photosynthetic carbon metabolism in cultured photoautotrophic cells. Proc. 5th Intl. Cong. Plant Tissue and Cell Culture, Plant Tissue Culture 1982. Tokyo, Maruzen.

Yasuda, T., T. Hashimoto, F. Sato and Y. Yamada. (1980). "An efficient method of selecting photoautotrophic cells from cultured heterogeneous cells." Plant Cell Physiol. 25(5): 929-932.

Zilkah, S., P. F. Bocion and J. Gressel. (1977). "Cell cultures vs. whole plants for measuring phytotoxicity II. correlations between phytotoxicity in seedlings and calli." Plant Cell Physiol. 18: 657-670.